American Journal of
Veterinary Research

CONTENTS

The Action of Adrenocorticotropic Hormone on Circulating Eosinophils in Dogs—A Proposed Screening Method for Evaluating Adrenal Cortical Function.....489
J. E. Martin, R. G. Skillen, and M. J. Deubler

The Structure and Histopathology of the Canine Meniscus.....495
S. Paatsama

The Teeth of the Ox in Clinical Diagnosis. III. Developmental Anomalies and General Pathology.....500
N. L. Garlick

The pH Values, Carbon Dioxide Content, and the Levels of Sodium, Potassium, Calcium, Chloride, and Inorganic Phosphorus of the Blood Serum of Normal Cattle.....509
B. J. McSherry and I. Grinyer

An Evaluation of the Complement-Fixation Test for Anaplasmosis.....511
K. E. Price, W. E. Brock, and J. G. Miller

Antibodies for Hemolysins of *Micrococcus Pyogenes* in Whey and Serum of Dairy Cattle.....517
J. Lasmanis and G. R. Spencer

An Outbreak of Histoplasmosis Involving Animals and Man.....520
R. W. Menges, M. L. Furcolow, and R. T. Habermann

Virus Diarrhea in Cattle.....525
J. A. Baker, C. J. York, J. H. Gillespie, and G. B. Mitchell

Studies on *Neoascaris Vitulorum*. III. Further Observations on Inoculation of Calves with Notes on Prenatal Infection.....532
P. G. Refuerzo and F. S. Albis-Jimenez

Disturbances in Acid-Base Balance and Electrolyte in Calf Diarrhea and Their Treatment. A Report of Eighteen Cases.....535
B. J. McSherry and I. Grinyer

Crystallization Patterns in Vaginal and Cervical Mucus Smears as Related to Bovine Ovarian Activity and Pregnancy.....542
J. F. Bone

The Survival of Pathogenic Bacteria in Bovine Semen Preserved by Freezing.....548
J. W. Macpherson and N. A. Fish

***Actinomyces Bovis* Isolated from Six Cases of Bovine Orchitis. A Preliminary Report**.....551
A. Kimball, M. J. Twiehaus, and E. R. Frank

(Continued on page 487)

A Publication of the American Veterinary Medical Association

Volume XV | OCTOBER, 1954 | No. 57

Instructions to Authors

AMERICAN JOURNAL OF VETERINARY RESEARCH

Exclusive Publication.—Articles submitted for publication are accepted with the understanding that they are not submitted to other journals, which is ethical publication procedure.

Manuscripts.—Manuscripts, including **footnotes, references,** and **tables,** must be typewritten, **double-spaced,** on 8½- by 11-in. **bond paper,** and the **original,** not the carbon copy, submitted. One-inch margins should be allowed on the sides, with 2 in. at top and bottom. Articles should be concise and to the point. Short, simple sentences are clearer and more forceful than long, complex ones.

Illustrations.—Photographs should be furnished in glossy prints, and of a size that will fit into the AMERICAN JOURNAL OF VETERINARY RESEARCH with a minimum of reduction. Photomicrographs which can not be reduced should be marked for cropping to 1-column or 2-column width. Identifying marks within the photomicrographs, such as arrows, letters, or numbers, should be clearly marked with black India ink or white opaque ink to insure good reproduction and must be large enough to stand reduction, if necessary. Drawings, graphs, and charts should be made clearly and accurately in India ink on white paper and a glossy print of them submitted when possible. Numbers or letters appearing on graphs or charts should be large enough to allow for any reduction necessary for the chart or graph to fit Journal pages. Blue lines in graph paper drop out in reproduction; therefore, if lines are required they must be drawn in black ink. All illustrations should bear the name of the author and the illustration number on the back.

Tables.—Tables should be simple and typed **double space.** Complex tables are not conducive to perusal. It is wiser to summarize complex material than to tabulate it.

References.—References should be typed **double space** and should be prepared in the following style: name of author, title of article, name of periodical with volume, year, and page numbers. References to journals not commonly known should give the complete name of the periodical, and where published so that they may be added to our reference files. When books are cited, the name of publisher, location, edition, and year should be given.

Editors

W. A. AITKEN, J. G. HARDENBERGH, HELEN S. BAYLESS; L. A. MERILLAT (editor emeritus).
Committee on Journal: S. F. SCHEIDY, A. H. QUIN, FLOYD CROSS.

Research Council

Anatomy and Histology

ROBERT GETTY, D.V.M., Ph.D. *(Secretary)*, Professor of Veterinary Anatomy and Histology, Division of Veterinary Medicine, Iowa State College, Ames, Iowa (1955).

Bacteriology (Immunology and Biological Therapy)

L. C. FERGUSON, D.V.M., Ph.D. *(Chairman)*, Department of Veterinary Science, Ohio Agricultural Experiment Station, Wooster, Ohio (1956).

Biochemistry and Animal Nutrition

M. J. SWENSON, D.V.M., Ph.D., Department of Physiology, School of Veterinary Medicine, Kansas State College, Manhattan, Kan. (1956).

Large Animal Medicine

E. F. EBERT, D.V.M., Head, Department of Medicine and Surgery, School of Veterinary Medicine, University of Missouri, Columbia, Mo. (1956).

Large Animal Surgery

A. G. DANKS, B.S., D.V.M., Professor, Department of Surgery, New York State Veterinary College, Cornell University, Ithaca, N. Y. (1957).

Parasitology

R. D. TURK, D.V.M., Head, Department of Veterinary Parasitology, School of Veterinary Medicine, Texas A. & M. College, College Station, Texas (1957).

Pathology

T. LLOYD JONES, D.V.M., M.Sc., Principal, Ontario Veterinary College, Guelph, Ont. (1956).

Physiology and Pharmacology

D. K. DETWEILER, V.M.D., M.S., Associate Professor of Pharmacology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa. (1956).

Poultry Pathology

C. A. BRANDLY, D.V.M., M.S., Chairman, Department of Veterinary Science, University of Wisconsin, Madison, Wis. (1957).

Radiology

MYRON A. THOM, D.V.M., Practitioner, 959 S. Raymond Ave., Pasadena, Calif. (1957).

Small Animal Medicine

J. C. FLINT, D.V.M., M.S., Practitioner, 1220 S. State St., Salt Lake City, Utah (1955).

Small Animal Surgery

J. E. GREENE, D.V.M., M.S., Professor of Surgery and Medicine, College of Veterinary Medicine, Alabama Polytechnic Institute, Auburn, Ala. (1955).

Veterinary Hygiene

ALFRED G. KARLSON, D.V.M., Ph.D., Associate in Experimental Pathology, Mayo Foundation, Rochester, Minn. (1955).

Virus Diseases

C. H. CUNNINGHAM, D.V.M., Ph.D., Professor of Bacteriology and Public Health, School of Veterinary Medicine, Michigan State College, East Lansing, Mich. (1955).

Member-at-Large

BURTON J. GRAY, D.V.M., P. O. Box 797, Fort Dodge, Iowa (1957).

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Additional entry as Second-Class Matter at the Post Office at Champaign, Illinois, approved February 17, 1953.

Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors.

Subscription price: $5.00 per year for United States and countries affiliated with the Pan American Postal Union; Canada and foreign countries, $5.30. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $2.50 per year added to the annual dues of $15.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

(Contents continued from cover)

Use of Rabbit-Passaged Strains of the Nakamura LA Rinderpest Virus for Immunizing Korean Cattle.....554
R. C. Reisinger, C. P. Mun, and N. S. Lee

A Study of Calcium in the Blood Serum of Egyptian Buffaloes and Native and Crossbred Cattle.....561
H. Reda and H. Salem

Control of Pasture-Acquired Infections of *Nematodirus Spathiger* and *Haemonchus Contortus* in Lambs with Phenothiazine-Salt Mixture.....564
J. H. Turner and M. L. Colglazier

Vaccination of Sheep with *Clostridium Perfringens* Type D Vaccines.....574
R. C. Percival, R. L. Burkhart, M. S. Cooper, and F. V. Martini

The Microscopic Anatomy of the Digestive Tract of *Sus Scrofa Domestica*.....578
M. W. Sloss

Laboratory Studies on Erysipelas. I. Immunization Against Swine Erysipelas and Susceptibility of Swine to Challenge.....594
M. S. Cooper, G. Personeus, M. J. Harvey, and R. C. Percival

Experimental *Vibrio Fetus* Infection in Guinea Pigs. III. Pathogenicity Studies and the Effect of Guinea Pig Passage upon Agglutinogens.....599
E. V. Morse and M. Ristic

The Secondary Immune Response in Chickens Revaccinated with Inactivated Newcastle Disease Virus Vaccine.....604
M. S. Hofstad

The Therapeutic Effect of Streptomycin on Infectious Coryza of Chickens Caused by *Hemophilus Gallinarum*. I. Blood Serum Levels of Streptomycin in Chickens..607
S. Bornstein and Y. Samberg

The Therapeutic Effect of Streptomycin on Infectious Coryza of Chickens Caused by *Hemophilus Gallinarum*. II. Isolation and Culture of *Hemophilus Gallinarum*, and Some of Its Biochemical Reactions.....612
S. Bornstein and Y. Samberg

***Corynebacterium Pyogenes*—A Cultural and Serological Study**.....617
J. F. Ryff and J. Browne

Summary of Toxicological Information on 2,4-D and 2,4,5-T Type Herbicides and an Evaluation of the Hazards to Livestock Associated with Their Use.....622
V. K. Rowe and T. A. Hymas

The Illustration of the Morphology of *Vibrio Fetus* by Electron Microscopy.....630
H. E. Rhoades

Marginal Punched Cards in Veterinary Research.....634
C. W. Schwabe and L. R. Davis

The Influence of Cobalt on the Action of Antibiotics.....639
J. C. Trace and G. T. Edds

The Effects of a Chlorophyll Derivative when Included in a Ration Fed Rats. II. Reproduction, Blood, and Tissue Studies.....643
E. F. Reber and D. A. Willigan

Response of the Cave Bat *(Myotus Lucifugus)* to the "B" Virus.....647
R. L. Reagan, E. C. Delaha, and A. L. Brueckner

DIFCO

preferred media
for isolation and differentiation
of

ENTERIC PATHOGENS

SALMONELLA — SHIGELLA

Isolation

Bacto-S S Agar
Bacto-MacConkey Agar
Bacto-Bismuth Sulfite Agar
Bacto-Brilliant Green Agar
Bacto-Selenite Broth
Bacto-Tetrathionate Broth Base

Differentiation

Bacto-Triple Sugar Iron Agar
Bacto-S I M Medium
Bacto-Purple Broth Base
Bacto-Purple Agar Base
Bacto-Urea Broth
Bacto-Urea Agar Base

ENTEROCOCCI

Bacto-Azide Dextrose Broth
Bacto-Azide Blood Agar Base
Bacto-S F Medium
Bacto-Phenylethanol Agar
Bacto-Enterococci Presumptive Broth
Bacto-Enterococci Confirmatory Broth
Bacto-Enterococci Confirmatory Agar

ENDAMOEBA HISTOLYTICA

Bacto-Endamoeba Medium
with Bacto-Horse Serum and Bacto-Rice Powder

THE DIFCO MANUAL, *NINTH EDITION*,
including descriptions of these media and their use,
is available on request.

Specify DIFCO — the trade name of the pioneers in the research and development of Bacto-Peptone and Dehydrated Culture Media

DIFCO LABORATORIES
DETROIT 1, MICHIGAN

American Journal of Veterinary Research

COPYRIGHT 1954 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

VOL. XV OCTOBER, 1954 No. 57

The Action of Adrenocorticotropic Hormone on Circulating Eosinophils in Dogs—A Proposed Screening Method for Evaluating Adrenal Cortical Function

JOHN E. MARTIN, V.M.D.; RALPH G. SKILLEN, B.A.;
M. JOSEPHINE DEUBLER, V.M.D., M.S., Ph.D.

Philadelphia, Pennsylvania

WITH THE increasing use of endocrine therapy in veterinary medicine, there is a need for clinical tests which will aid in the diagnosis of endocrine diseases in domestic animals. It is our purpose to present here a simple diagnostic test that is used in human medicine as a screening method for evaluating adrenal cortical function, a procedure which we feel will be useful for this purpose in the dog.

In man and in various laboratory animals, administration of adrenocorticotropic hormone (ACTH) leads to a marked decrease in circulating eosinophils.[5, 8, 17] The exact cause of the decrease is not known, but it has been established that it is related to an increased secretion of adrenal cortical hormone (or hormones) by the adrenal cortex as a result of stimulation by ACTH. In Addison's disease of man, a condition in which adrenal cortical function is essentially lost, little or no fall of circulating eosinophils occurs after injection of ACTH.[5, 8, 17] However, if a suitable adrenal cortical hormone is administered to individuals with this disease (or to normal individuals) an eosinopenia occurs.[5, 8, 17] In addition to the fall in eosinophils, there is a concurrent decrease in circulating lymphocytes and an increase in neutrophils following administration of ACTH or one of the suitable adrenal cortical hormones in man.

In laboratory animals (mice, rats, and rabbits) with intact adrenal glands, but not in adrenalectomized animals, injection of ACTH leads to an eosinopenia and lymphopenia similar to those described for man.[3, 15] In dogs, Reinhardt *et al.*[12] have shown that subcutaneous injection of 10 mg. of ACTH resulted in a lymphopenia within six hours.

Since there appears to have been no detailed studies dealing with the effect of ACTH on circulating eosinophils in normal or diseased dogs, we decided to investigate the problem in this species using animals maintained under clinical conditions.

METHODS

Adrenocorticotropic hormone was employed in two forms.* One form contained 10 international units (I.U.) per cubic centimeter of ACTH in a gelatin vehicle containing 30 per cent propylene glycol. The second type contained 40 I.U. of ACTH in a gelatin vehicle containing 0.5 per cent phenol. The preparations are standardized so that 1 I.U. is equivalent to 1.0 mg. of ACTH.

The effect of ACTH on circulating eosinophils was studied in the following manner. Between 9 a.m. and 10 a.m. a blood sample of approximately 1.0 cc. was taken from the animal to be tested. The blood was drawn in a heparinized syringe from the cephalic vein. Heparin is the preferable anticoagulant since it has been observed that oxalate results in a rather rapid destruction of dog eosinophils. Immediately after taking the blood sample, a dose of ACTH was injected intramuscularly. A direct eosinophil count was made on each blood

Assistant professor of physiology and pharmacology (Martin); senior student (Skillen); and assistant professor of veterinary pathology (Deubler), School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

* ACTH used in these studies was generously supplied by the Veterinary Research Division, The Armour Laboratories, Chicago, Ill.

sample within one hour after it was drawn, and this was designated as the initial or control count. At varying intervals after the injection of ACTH, additional heparinized blood samples were taken and eosinophil counts were made.

The following technique was employed in making counts. The heparinized blood sample was thoroughly mixed by inverting several times the tube in which it was contained. Blood was drawn up to the 1 mark in a standard white-cell-counting pipette. A special diluting fluid was then drawn up to the 11 mark on the pipette. A second pipette was filled in the same manner, and both were shaken for two minutes. Each pipette was used to fill two hemocytometers; that is, each pipette was used to fill all four counting chambers on its respective hemocytometers or eight counting chambers in all. Before the chambers were filled, three drops were discarded from the pipette so that only diluting fluid which had been admixed with blood would be used. After waiting fifteen minutes, the cells in all nine squares of each chamber were counted. The number of eosinophils was calculated using the following formula: average count per chamber x 11.1 = number of eosinophils per cubic millimeter. The percentage of change in eosinophil count in blood samples taken after administration of ACTH was computed using the formula:

$$100 - \frac{\text{Eosinophils/mm.}^3 \text{ after ACTH}}{\text{Eosinophils/mm.}^3 \text{ before ACTH}} \times 100$$

The diluting fluid employed in making eosinophil counts was described by Pilot.[10] It is prepared as follows:

Propylene glycol	50 ml.
Distilled water	40 ml.
1% phloxine in distilled water (stock solution)	10 ml.
10% sodium carbonate (stock solution)	1 ml.

The ingredients are mixed with special care taken to prepare and measure the sodium carbonate solution in an accurate manner. Then the solution is filtered. The final solution is stable at room temperature for at least one month. It has the property of lysing all blood cells except the eosinophils which are stained a deep red and stand out clearly in the counting chamber. The fifteen-minute wait after mixing the solution in the pipettes is to allow complete lysis of other cells. For this, the sample may be allowed to remain in the pipette or it may be placed in the counting chamber. If left in the pipette during this period, frequent shaking is advisable.

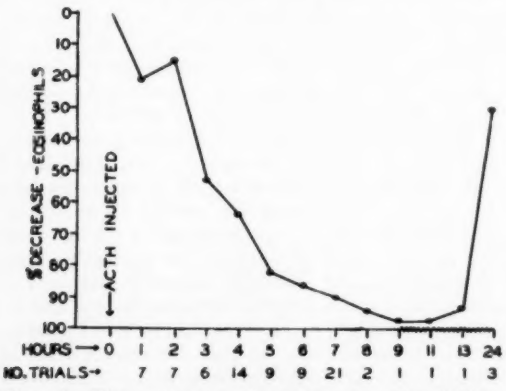


Fig. 1—The average decrease in circulating eosinophils at various hours after intramuscular administration of ACTH in doses of 5 to 50 I.U.

RESULTS

Results are shown in table 1 and in figures 1 and 2.

A total of 33 separate experiments on 18 different dogs were conducted (table 1). Dosage of ACTH varied from 5.0 to 50.0 I.U. intramuscularly. In 5 cases, no ACTH was administered and counts were made at various intervals. There appeared to be no significant difference in the response of circulating eosinophils to either maximum or minimum dosage of ACTH employed and therefore, after a few initial experiments, a dose of 10 I.U. was selected for testing the eosinophil response in the majority of animals. A relatively consistent decrease in circulating eosinophils occurred with this dose regardless of the weight of the animals which varied, in these experiments, from 12 to 65 lb.

Initial eosinophil counts varied considerably among different animals and also in the same animals when made at different times. The range of initial counts was from 10 to 1,285 eosinophils per cubic millimeter. Nine animals listed in table 1 (3, 4, 5, 7, 8, 9, 10, 11, and 16) were suffering from some disease or had undergone surgery shortly before being used in this study, and in general their initial eosinophil counts were lower than in the other dogs (1, 2, 6, 12, 13, 14, 15, 17, and 18) which were considered to be normal. The average of 12 initial eosinophil counts made on the diseased dogs and on the dogs which had undergone surgery was 281, whereas the average for normal animals was 735 per cubic millimeter. Thirteen different eosinophil counts were made on dog 1. The initial eosinophil counts for this animal varied from 588 to 1,035 eosinophils per cubic millimeter with the average being 778 per cubic millimeter. The possible significance of these variations will be discussed later.

In all of the 28 experiments in which ACTH was administered, a marked decrease in circulating eosinophils occurred (table 1). When the change in the number of eosinophils is expressed in percentage, there appears to be no relation to the initial count. In general, the same magnitude of decrease in eosinophils is observed after ACTH administration whether the initial count be high or low. In the five trials in which no ACTH was administered, there was little or no change in eosinophils when counts were made at seven-hour intervals.

The rate of fall of circulating eosinophils at various hours after the administration of ACTH is shown in figure 1. The greatest number of counts were made at the seventh hour after the administration of ACTH since in most animals the number of circulating eosinophils had reached a very low level at this time. The average decrease in eosinophils at the seventh hour for all doses of ACTH was 91 per cent with a range of 75 to 99 per cent (table 1). Between the seventh and ninth hours there is a further tendency for the cells to decrease in number, but the observations made to date after seven hours are too few to be of any significance. In 3 cases counts were made twenty-four hours after administration of ACTH, and at this time the number of eosinophils in all animals tested was returning toward the initial level.

One objective of this study was to determine the minimum dose of ACTH which would produce a maximum fall in circulating eosinophils in the dog. While a dose of 5.0 I.U. of ACTH resulted in a marked decrease of eosinophils in the 3 cases in which it was employed (table 1), a dose of 10 I.U. was used in most animals since it appeared that this dose might be more suitable for the wide weight ranges encountered in various breeds of dogs. A 10-mg. dose was used in 19 trials on 14 animals in which counts were made up to at least seven hours after administering the hormone (fig. 2). For comparison, this group is divided into those exhibiting some disease process and those which were considered normal. When calculated as percentage of the initial count, the average

TABLE 1—Circulating Eosinophil Response in Dogs to Various Doses of Adrenocorticotropic Hormone (ACTH)

Animal (No.)	Exper. (No.)	Wt. (lb.)	Dose ACTH (I.U.)	Eosinophil count (cells/mm.³)		Eosinophils % change
				Before ACTH	After ACTH*	
1	1	65	None	1,022	1,034 (7)	+ 1
	2		None	983	883 (7)	− 10
	4		50	1,035	205 (6)	− 80
	5		50	719	33(13)	− 95
	6		25	705	17 (7)	− 98
	8		15	674	28 (6)	− 96
	9		5	599	86 (6)	− 86
	12		10	922	15(11)	− 98
	13		10	588	14 (7)	− 98
	17		10	602	86 (5)	− 86
	25		None	733	610 (7)	− 16
	28		10	838	11 (7)	− 99
	31		10	688	145 (7)	− 79
2	3	40	None	736	638 (7)	− 13
3	7	25	25	128	11 (8)	− 92
4	10	25	5	103	1 (7)	− 99
5	11	25	10	233	25 (6)	− 89
6	14	50	10	691	16 (7)	− 98
7	15	30	10	1,080	91 (7)	− 92
7	26		10	242	44 (7)	− 82
8	16	20	10	78	7 (7)	− 92
9	18	18	10	403	47 (7)	− 88
9	19		None	392	392 (7)	0
10	20	50	10	209	5 (7)	− 98
10	29		10	325	17 (7)	− 95
11	21	12	10	172	43 (7)	− 75
12	22	45	10	322	6 (7)	− 98
13	23	65	10	872	42 (7)	− 95
14	24	50	10	372	4 (7)	− 99
15	27	35	10	90	15 (7)	− 83
16	30	18	10	10	2 (7)	− 80
17	32	50	10	1,285	247 (7)	− 81
18	33	12	10	253	6 (7)	− 98

* Figures in parentheses indicate hours after ACTH that eosinophil count was made.

fall in circulating eosinophils at the seventh hour was 87 per cent for the former group and 92 per cent for the latter. The results of the five trials in which no ACTH was administered are also shown in figure 2.

DISCUSSION

The decrease in circulating eosinophils following administration of ACTH is related to an increased secretion of adrenal cortical hormone. However, the exact mechanism responsible for this change is not known. Reinhardt and Li [13] reported that injection of ACTH in rats resulted in a rapid decrease in the number of lymphocytes appearing in the thoracic duct lymph and postulated that the cause was a decreased outpouring of these cells from the thymus and lymph nodes. It is possible that there is also a decreased outpouring of eosinophils from the bone marrow. Muehrke *et al.*[9] incubated defibrinated blood with cortisone and found a highly significant fall in eosinophils within four hours. In other work, Feldman [4] found that addition of lipo-adrenal cortex to blood samples caused a rapid and complete destruction of lymphocytes. These studies would seem to indicate that one possible explanation for the eosinopenia and lymphopenia following the administration of ACTH or adrenal cortical hormone is that the latter compounds cause a lysis of the cells. It appears that only adrenal cortical hormones of the 11-oxysteroid type, of which cortisone (11-dehydro-17-hydroxy-corticosterone) is representative, are responsible for the decline in eosinophils. Godlowski [6] is of the opinion that the 11-oxygenated corticosteroids accelerate intracellular proteolysis which leads to the lysis of these cells. Desoxycorticosterone, a member of the 11-desoxy group of adrenal steroids, does not produce a similar decrease in the number of circulating eosinophils.

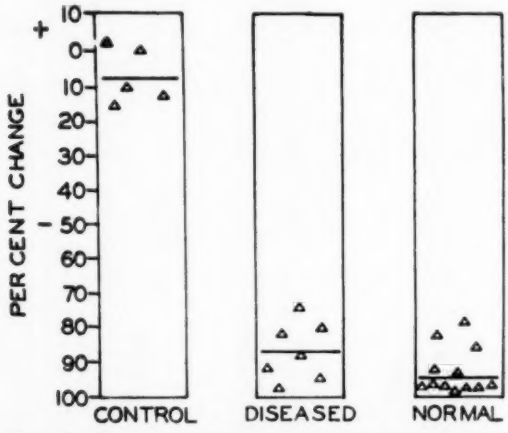


Fig. 2—The percentage of change in eosinophils seven hours after injecting 10 mg. of adrenocorticotropic hormone.

The control group received no ACTH; the diseased group included a variety of diseases none of which are thought to be associated with an adrenal cortical dysfunction; the normal group included dogs showing no obvious signs of disease.

In addition to the injection of ACTH or one of the suitable adrenal cortical preparations, many other agents or conditions leading to increased activity of the adrenal cortex result in a fall in circulating eosinophils. Examples of these in man are major surgery,[14] excessive heat or cold,[16] injection of insulin,[7] pregnancy, labor, and eclampsia.[2] There are numerous reports on the action of epinephrine as an agent which produces an eosinopenia and a lymphopenia.[11,15] In dogs, injection of 0.3 to 1.0 mg. of epinephrine subcutaneously leads to a 50 per cent fall in eosinophils within four hours.[11]

Extreme variations in the initial eosinophil count observed in different dogs employed in this study may reflect differences in their levels of adrenal cortical activity at the time the tests were carried out. Dog 30 was suffering from congenital heart disease (suspected interventricular septal defect) and exhibited frequent bouts of cyanosis. It can be supposed, therefore, that the animal was in a more or less chronic state of stress, which may account for the very low initial eosinophil count (10/cu. mm.). The initial eosinophil count may prove to be of some diagnostic significance since in severely ill animals one would expect a low level of eosinophils if there is sufficient adrenal cortical reserve. Coffin and Munson [1] reported the presence of an eosinopenia and a lymphopenia in dogs suffering from Cushing's syndrome, and we have observed an eosinopenia in a dog with an adrenal tumor. In both conditions, there is produced an excess of adrenal cortical hormone.

In man, the circulating eosinophil response to an injection of ACTH is employed as a test for evaluating adrenal cortical reserve. The test consists of making a direct eosinophil count immediately

before and four hours after the intramuscular injection of 25 I.U. of ACTH. A fall in eosinophils of 50 per cent or more usually indicates a satisfactory adrenal cortical response. Thorn [17] states that if the fall in eosinophils fails to occur, a complete absence of adrenal cortical reserve may be assumed. Conversely, a significant decrease in circulating eosinophils excludes Addison's disease. In man, it has been determined that the initial level of eosinophils does not affect the percentage fall except during the height of allergic eosinophilia when the production of these cells exceeds their disappearance.

The study reported here forms the basis for a method to evaluate adrenal cortical reserve in dogs. Obviously, before this method can be accepted as an established diagnostic aid, it will be necessary to employ it in clinical cases where it can be established definitely by other critical tests (chemical and/or bioassay) that a functional deficiency of the adrenal cortex exists. Presumably, cases of adrenal cortical deficiency are rare in dogs, but it may well be that they have not been recognized because of a lack of suitable diagnostic methods. The experiments reported here indicate that in normal dogs, or in dogs suffering from diseases in which there is no reason to suspect an adrenal cortical deficiency, the circulating eosinophil response to ACTH is essentially similar to that observed in man. Therefore, in its present form, the described procedure should be of value as a clinical screening method for evaluating adrenal cortical reserve in dogs. A fall in circulating eosinophils of 70 per cent or more within seven hours after administering ACTH is presumably indicative of satisfactory adrenal cortical reserve in dogs. If a fall of less than 70 per cent is obtained, the procedure should be repeated to eliminate the possibility of technical errors or other transient factors which may alter the eosinophil response. Although no opportunity has arisen to employ this procedure in a known clinical case of adrenal cortical deficiency, we feel that less than a 70 per cent fall within seven hours would be evidence of such a deficiency. Like all clinical diagnostic procedures, this particular test must be correlated with other clinical findings and, if possible, with other more critical tests. In severe stress (e.g. infectious diseases), a normal adrenal cortex might be temporarily depleted in which case no eosinopenia would occur in response to the injected ACTH. Possibly such a finding would indicate a need for adrenal hormone replacement therapy. However, the clinical history should differentiate such a temporary depletion from a chronic adrenal insufficiency.

Other situations in which the eosinophil count may not decrease following ACTH injection are those of certain cases of allergy and parasitism. In these cases, there may be a marked eosinophilia, with the production of cells exceeding the ability of cortical hormone to destroy them. In 2 dogs (not reported here), a marked rise in eosinophils occurred within two hours after administration of ACTH. The eosinophil level later fell below the initial count, but even at eight hours after the injection of ACTH a 70 per cent decrease in the eosinophil count had not been reached. These cases may reflect an allergic reaction to ACTH which is protein in nature. However, neither animal exhibited any other signs of such a reaction (e.g. urticaria, dyspnea, or facial edema).

Summary

Injection of 5 to 50 international units of adrenocorticotropic hormone (ACTH) in normal dogs, or in dogs suffering from diseases in which no adrenal cortical deficiency is known to exist, resulted in a 75 to 99 per cent decrease in circulating eosinophils within seven hours. It is suggested that the circulating eosinophil response to ACTH may be useful as a screening method for evaluating adrenal cortical reserve in dogs.

References

[1] Coffin, D. L., and Munson, T. O.: Endocrine Diseases of the Dog Associated with Hair Loss. J.A.V.M.A., *123*, (1953): 402.

[2] Davis, M. E., and Hulit, B. E.: Changes in Circulating Eosinophils in Women During the Menstrual Cycle and Reproduction. J. Clin. Endocrinology, *9*, (1949): 714.

[3] Dougherty, T. F., and White, A.: Influence of Hormones on Lymphoid Tissue Structure and Function. The Role of Pituitary Adrenotropic Hormone in the Regulation of the Lymphocytes and Other Cellular Elements of the Blood. Endocrinology, *35*, (1944): 1.

[4] Feldman, J. D.: The *In Vitro* Reaction of Cells to Adrenal Cortical Steroids with Special Reference to Lymphocytes. Endocrinology, *46*, (1950): 552.

[5] Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G.: Clinical Studies with Pituitary Adrenocorticotropin. J. Clin. Endocrinology, *8*, (1948): 15.

[6] Godlowski, Z. Z.: Enzymatic Concept of Anaphylaxis and Allergy. E. & S. Livingstone Ltd., Edinburgh and London, (1953): 60-104.

[7] Godlowski, Z. Z.: Eosinopenia Caused by Adrenalin Infusion and by Insulin Hypoglycemia. Brit. Med. J., *1*, (1948): 46.

[8] Hills, A. G., Forsham, P. H., and Finch, C. A.: Changes in Circulating Leukocytes Induced by the Administration of Pituitary Adrenocorticotropic Hormone (ACTH) in Man. Blood, *3*, (1948): 755.

[9] Muehrcke, R. C., Lewis, J. L., and Kark, R. M.: The Effects of Cortisone and Heparin on Eosinophils in Defibrinated Human Blood. Science, *115*, (1952): 377.

[10] Pilot, M. L.: Use of Base in Fluids for Counting Eosinophils. Am. J. Clin. Path., *20*, (1950): 870.

[11] Recant, L., Hume, D. M., Forsham, P. H., and Thorn, G. W.: Effect of Epinephrine on the Pituitary Adrenocortical System. J. Clin. Endocrinology, *10*, (1950): 187.

[12] Reinhardt, W. O., Aron, H., and Li, C. H.: Effect of Adrenocorticotrophic Hormone on Leukocyte Picture of Normal Rats and Dogs. Proc. Soc. Exptl. Biol. and Med., *57*, (1944): 19.

[13] Reinhardt, W. O., and Li, C. H.: Depression of Lymphocyte Content of Thoracic Duct Lymph by Adrenocorticotrophic Hormone. Science, *101*, (1945): 360.

[14] Roche, M., Hills, A. G., and Thorn, G. W.: The Level of Circulating Eosinophils as an Indicator of Adrenal Cortical Adequacy Following Major Surgery. J. Clin. Endocrinology, *9*, (1949): 662.

[15] Speirs, R. S., and Meyer, R. K.: The Effects of Stress, Adrenal and Adrenocorticotrophic Hormones on the Circulating Eosinophils of Mice. Endocrinology, *45*, (1949): 403.

[16] Stein, H. J., Bader, R. A., Eliot, J. W., and Bass, D. E.: Hormonal Alterations in Men Exposed to Heat and Cold Stress. J. Clin. Endocrinology, *9*, (1949): 592.

[17] Thorn, G. W., Forsham, P. H., Prunty, F. T. G., and Hills, A. G.: A Test for Adrenal Cortical Insufficiency. J. Am. M. A., *137*, (1948): 1,005.

The Structure and Histopathology of the Canine Meniscus

S. PAATSAMA, D.V.M.

Helsinki, Finland

The Structure of the Meniscus

Survey of Literature.—As early as 1905, Retterer [9] investigated the structure of the menisci in man and in domestic animals. He pointed out that the phase of formation of cartilage in sheep, for instance, occurs at the postnatal age of 2 to 3 months, which corresponds to the age of 5 years in man. According to Retterer, the fibrous structure of the menisci is the same in domestic animals as in man, the inner part, the fibrocartilaginous-collagenous part, containing encapsulated cells. Both surfaces of the meniscus consist of collagenous elastic lamellae containing vesiculated cells more or less encapsulated.

In 1923, Ischido [4] added a significant contribution to the knowledge of the menisci in man. The meniscus of the newborn child consists of three parts, an external zone of connective tissue with numerous blood vessels, a middle fibrous zone without blood vessels, and an inner zone with numerous cells and scanty intercellular substance. When growth begins, the cells of the middle zone decrease in number, the amount of intercellular substance increases, and traces of fibrocartilage are found. Later the blood vessels disappear even from the external zone; the structure then conforms to that of fibrocartilage.

Szymonowicz [12] and Stöhr-Möllendorff,[11] in their textbooks of histology, considered that the menisci consist of connective tissue containing round cells. Rauber-Kopsch [7] regarded the menisci as formed from bundles of connective tissue and elastic fibers, with a thin cover of fibrocartilage on both surfaces.

In 1937, Schwerdtfeger [9] observed that in puppies the blood vessels in the external vascular zone are clearly distinguishable and radiate into the inner parts of the meniscus but do not reach the surface. As these blood vessels disappear, the meniscus changes into fibrocartilage.

Material and Investigation.—The material consisted of 30 dogs (17 experimental and 13 which had been destroyed in the surgical clinic of the Royal Veterinary College in Stockholm, Sweden). Included were dogs of different breeds and ages, the youngest being 2 days old, the oldest 13 years.

The meniscus of a 2-day-old puppy consists of connective tissue, the peripheral part being attached to the capsule and deriving its blood supply from it. The bundle structure of the middle part of the meniscus becomes visible during the period of growth, and at the same time the blood vessels disappear. The surfaces and the inner border of the meniscus become homogeneous, and the hyalinization of the inner border increases with age and seems to have importance as an elastic lamella in the function of the meniscus.

In the inner part of the meniscus of full-grown dogs, the collagen fibers are arranged in undulating fiber bundles running parallel to the peripheral border and following the shape of the meniscus from insertion to insertion. The undulating structure of the inner part seems to give to the meniscus its functional elasticity. The bundles stain evenly, and scanty metachro-

Fig. 1—The left stifle joint of an 8-year-old Rottweiler showing multiple tears of both menisci; a total rupture of the anterior cruciate ligament which is almost entirely absorbed; osteoarthritis showing defects of the cartilages, excrescences at the outer border of the femoral condyles, at the border of the tibia, and of the patella; synovitis and thickened fibrous capsule.

From the Veterinary Medical Institute of the University of Helsinki, Finland. Investigations were made in the Royal Veterinary College, Stockholm, Sweden.

matic tissue of chromotropic substance, which forms stripes in the fiber structure, is easily discerned between them.

THE HISTOPATHOLOGY OF THE MENISCUS

Survey of Literature.—The histopathology of the menisci in man has been investigated by Ischido,[4] Mandl,[5] Tobler,[13] Bircher,[2] Wallinheimo,[14] and others. These investigations revealed that fatty degeneration is the most common pathological change and that it may begin early. The myxomatous degeneration or "schleimige degeneration" also begins early in man, and it involves a gradual decomposition of the structure with a tendency toward the assimilation of calcium. The transformation of fibrocartilage into hyaline cartilage is more rare.

In older individuals, the different forms of degeneration are usually combined. The pathological changes are often primary, that is, independent of the state of the other parts of the joint. However, if the joint has been injured the pathological changes have to be regarded as the consequence of the derangements of the other parts.

Through the repeated formation of fissures, the meniscus may collapse and tear without any trauma. In the periphery, which is subject to less pressure, there is not such a pronounced tendency to form fissures. Instead, cysts occur in this zone, as the result of a degenerative process.

Fig. 2—Hyalinized areas in the anterior horn of the medial meniscus of a 4-year-old German Drahthaar Pointer, eight weeks after trauma. Van Gieson stain. x 78.

Fig. 3—Colliquative necrosis in the periphery of the meniscus of an Alsatian, 13 years old. Foot and Foot silver impregnation. x 78.

Schwerdtfeger[9] observed in the dog that from the third year on there is a disintegration of the nuclei. From the seventh year on, degeneration of the nuclei and necroses of the cartilage cells take place, and hyalinization and calcification zones appear in the horns and the borders of the menisci. These increase with age.

Material and Investigation.—The material consisted of 58 dogs, including 28 operated upon in the surgical clinic for internal derangements of the stifle joint, and the 30 dogs mentioned previously. These dogs were used to investigate the development of osteoarthritis caused by cutting the cruciate ligament or the development of pathological changes as a result of cutting the meniscus or removing it entirely. The specimens used were from the whole menisci, except in the cases of the dogs which had undergone surgery where they were collected from the removed part of the menisci. For the examination, the menisci were cut transversely and longitudinally. All the slides were stained by the method of van Gieson, some were also stained with hematoxylin-eosin, thionine, Sylven's toluidine blue, Ladevigs stain, and the silver-impregnation of Foot and Foot. Furthermore,

some of the slides were stained with Scharlach rot for fat, Bock's calcium stain, and Weigert's elastic stain.

A detailed analysis of the menisci of 20 dogs (which are chronologically the first of 37 dogs examined during 1951) operated upon for internal derangements of the stifle joint is given in table 1. This degenerative arthritis is most typical in certain breeds, Scottish Terrier, Newfoundland, Boxer, and Rottweiler. The investigation by the author, in 1952,[6] demonstrated that, after pathological changes, the anterior cruciate ligament ruptured totally or partially, permitting subluxation movement and causing the lesions to develop in the menisci, collateral ligaments, articular cartilages, and capsule. Single or multiple longitudinal tears, but seldom a transverse tear, occur in the menisci. Through the strain of the joint the destructive picture of a gradually developing osteoarthritis becomes more marked, as is shown in figure 1.

The hyalinization of the inner border was found to have increased, and hyalinized areas were also found inside the border part, these areas differing sharply from their surroundings. Extensive hyaliniza-

Fig. 4—Fissure in the border areas of the meniscus of an Alsatian, 13 years old. Foot and Foot silver impregnation. x 100.

TABLE 1—A Detailed Analysis of the Menisci of 20 Dogs Operated Upon for Lesions of the Stifle Joint

No.	Breed	Age	Lameness	Macroscopic finding					Microscopic finding				
		Years	Duration (weeks)	Medial meniscus	Lateral meniscus	Ant. cruciate ligament	Post. cruciate ligament	Osteoarthritis	Hyalinization	Colliquative necrosis	Chromotropic subst.	Slit formation	Fatty degeneration
1	Boxer	4	2	l.t.	l.t.	tot.	part.	+++	++	+	++	++	
2	Rottweiler	2	4	m.l.t.	—	part.	—	+	++	—	+++	++	
3	Alsatian	6	24	—	m.l.t.	part.	—	+++	++	+	+++	+	—
4	Boxer	5	12	—	—	tot.	—	+++	++	+	++	++	
5	Rottweiler	2	4	—	p.t.	part.	—	++	+	—	+	+	
6	Boxer	6	2	p.t.	—	tot.	part.	+	++	—	++	++	
7	Newfoundland	2	36	m.l.t.	—	tot.	—	++	+	+	+	+	
8	Alsatian	7	4	p.t.	—	part.	—	+	++	+	++	+	+
9	Vorsteher	4	3	p.t.	—	tot.	part.	++	+	—	+	+	
10	Alsatian	8	6	m.l.t.	—	tot.	—	++	++	—	++	++	
11	Airedale Terrier	4	8	m.l.t.	tr.t.	tot.	—	+++	++	+	++	++	
12	Riesenschnauzer	6	21	m.l.t.	p.t.	tot.	—	+++	+	—	++	+	—
13	Spaniel	10	8	—	—	tot.	—	++	+++	+	+++	++	+
14	Rottweiler	8	8	—	p.t.	tot.	—	+++	++	+	+++	++	
15	Riesenschnauzer	6	8	m.l.t.	—	part.	part.	++	++	—	++	++	—
16	Newfoundland	4	50	l.t.	—	tot.	part.	+++	++	+	++	++	
17	Alsatian	10	1	p.t.	—	tot.	part.	++	+++	+	+++	++	
18	Newfoundland	3	20	p.t.	—	tot.	part.	+++	+	—	+	+	—
19	Newfoundland	5	8	m.l.t.	p.t.	tot.	part.	+++	++	+	++	++	
20	Airedale Terrier	8	1	m.l.t.	—	tot.	—	+++	+++	+	+++	++	+

l.t.—longitudinal tear; m.l.t.—multiple longitudinal tears; p.t.—peripheral tear; tr.t.—transverse tear; tot.—total rupture; part.—partial rupture.
+ slight alteration; ++ medium alteration; +++ severe alteration.

tion was observed in 3 cases (13, 17, and 20) all of which were old dogs (fig. 2). In many cases, hyalinization occurred together with a colliquative necrosis. In hyalinized areas, karyolysis was observed.

Fig. 5—Fatty degeneration and longitudinal tear in the meniscus of a 9-year-old Spitz. Scharlach rot. x 78.

Less often, the border area showed pathological changes; if these changes reached the peripheral border, which is rich in blood vessels, colliquative necrosis developed. This was found in 12 cases (fig. 3). In all cases, chromotropic substance occurred as irregularly dispersed areas, which often followed the shape of the tears. It was observed in parallel stainings that chromotropic substance occurred in the hyalinized areas. Often in the metachromatic areas there were increased numbers of nuclei, which differed from the normal by their more rounded form. The amount of chromotropic substance was slight in 4 cases, medium in 11, and abundant in 5.

The disappearance of the collagen fiber bundles and the formation of slits were noted in all cases. The slit usually followed the fiber structure, which is longitudinal. In the hyalinized areas of the borders, slit formation was often found (fig. 4).

Fatty degeneration was usually encountered only in connection with other changes in old dogs. It consisted of diffuse infiltrations of small and large fat droplets. In 3 of 7 cases, fatty degeneration was observed (fig. 5).

Calcification was not observed with other changes of the menisci in the dogs which had undergone surgery except in 1 case where calcification of the capsule had spread to the periphery of the meniscus. This case does not appear in table 1.

The histopathological investigation of the menisci of the dogs destroyed revealed changes, such as increased hyalinization, an increase in the amount of chromotropic substance, and even colliquative necrosis and slit formation. If there were lesions of the ligaments, the microscopic changes were also considerable. Changes were greater in the older dogs.

In the experimental dogs, which were originally healthy, the incisions made in the menisci did not cause any pathological changes. But severing the cruciate ligament, causing functional disturbances,

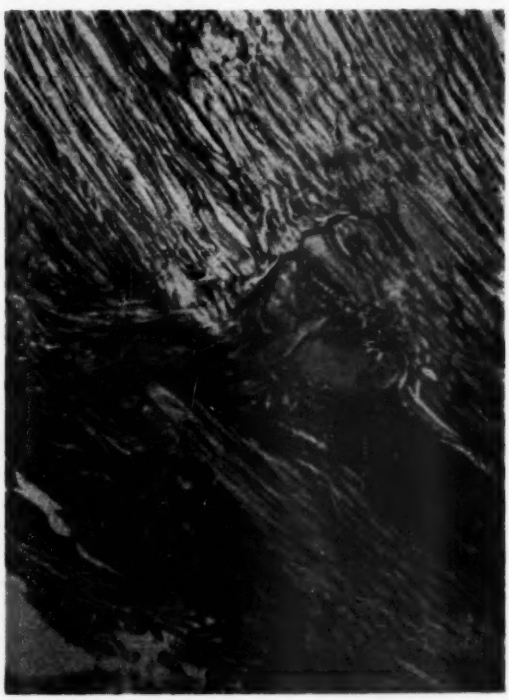


Fig. 6—The anterior cruciate ligament cut ten weeks earlier by changes of the medial meniscus of a 3-year-old Alsatian. Thionine. x 78.

gave rise to degenerative changes and osteoarthritis in the joint. In fifty days pathological changes, such as increased hyalinization, an increase in the amount of chromotropic substance, colliquative necrosis, and slit formation, were to be found (fig. 6). Consequently, the histopathological picture typical of the dogs which had undergone surgery made its appearance in the menisci of the experimental dogs also. The examination indicated that big dogs developed the pathological changes in a much shorter time than did the smaller ones.

Discussion

1) The investigations of Tobler and others have indicated that there occurs in the menisci myxomatous "schleim" degeneration. On the basis of his experiments on dogs, Ssolowjew [10] stated that the regeneration of connective tissue is associated with the appearance of great amounts of mucoid connective tissue. Thus, his opinion is the direct opposite of the old conception of "schleim" degeneration. Holmgren [3] pointed out that the chromotropic substance is associated with elastic structure and function. According to Tobler, the "schleim" degeneration in man, which begins early, is later usually combined with other degenerations. But this was not found to be the case in this investigation, and the increase of chromotropic substance may be regarded as being associated with the regeneration of the tissue in the areas where slit formation and other pathological changes occur.

2) The investigation, by means of multiple stainings, indicated that a relationship exists between chromotropic substance and hyalinization. The investigation of Altshuler and Angevine has indicated that the formation of the metachromatic material is closely related to processes which have customarily been designated as hyalinization. They state that the exact relationship and differences are not known.

3) The formation of slits of the menisci was observed in old dogs, especially where lesions of the ligaments had occurred. The mode of development of the tears appears to be such that they are formed from small, originally microscopic slits. This conforms with the clinical finding, longitudinal tears being most common.

4) The opinion of Tobler is that degeneration of the human meniscus promotes the formation of tears. The same pathological picture, however, is not to be found in the canine meniscus, partly perhaps because of the shorter life span of dogs and partly because the meniscus is not called upon to bear as much strain as in man. When lesions of the ligaments occur, however, degeneration takes place rapidly, and this leads to slit formation and tears of the menisci.

Summary

The author gives a short survey of the structure of the canine meniscus. The histopathology of the meniscus shows that pathological changes, such as increase of chromotropic substance and hyalinization, slit formation, fatty degeneration, and colliquative necrosis, are to be found in old dogs, particularly in cases of joint injuries. Cutting the cruciate ligament causes the changes to develop within fifty days.

References

[1] Altshuler, H., and Angevine, M.: Histochemical Studies on the Pathogenesis of Fibrinoid. Am. J. Path., *25*, (1949): 1061.

[2] Bircher, E.: Die Binnenverletzungen des Kniegelenks. Schweiz. med. Wchnschr., *2*, (1929):1292, 1309; Arch. klin. Chir, 1933.

[3] Holmgren, H. J.: Studien über Verbreitung und Bedeutung der Chromotropensubstanz. Ztsch. mikr.-anat. Forsch., *47*, (1940): 489.

[4] Ischido, B.: Über den Kniegelenksmeniskus. Virchows Arch. Path. Anat., *244*, (1923): 429.

[5] Mandl, F.: Zur Pathologie und Therapie der Zwischenknorpelerkrankungen des Kniegelenks. Arch. klin. Chir., *149*, (1927): 1461.

[6] Paatsama, S.: Ligament Injuries in the Canine Stifle Joint. Diss., Univ., Helsinki, Finland, 1952.

[7] Rauber-Kopsch: Lehrbuch der Anatomie des Menschen, 1932.

[8] Retterer, E.: Histogenèse de la vertebr. cartilagineuse de mammifères. De la structure des ménisques inter-articulaires de genou de quelques grands mammifères. Ibidem 1905.

[9] Schwerdtfeger, H.: Altersveränderungen am Kniegelenk des Hundes. Diss., Univ., München, Germany, 1937.

[10] Ssolowjew, A.: Experimentelle Untersuchungen über die chromotrope Grundsubstanz der Arterienwand. Virchows Arch. Path. Anat., *261*, (1926): 253.

[11] Stöhr-Möllendorff: P. H. Stöhrs Lehrbuch der Histologie, Gustav Fischer, Jena, 1933.

[12] Szymonowicz: Lehrbuch der Histologie, Kurt Kubizsch, Leipzig, 1930.

[13] Tobler, T.: Makroskopische und histologische Befunde am Kniegelenksmeniscus in verschiedenen Lebensaltern. Schweiz. med. Wchnschr., *2*, (1929): 1359; Zur normalen und pathologischen Histologie des Kniegelenkmeniscus. 57. Tagung Deutsch. Gesell. Chir., 1933.

[14] Wallinheimo, L. O.: Über die embryonale Entwicklung, Histologie und Degenerationserscheinungen der Menisken des Kniegelenks. Diss., Univ., Helsinki, Finland, 1938.

The Teeth of the Ox in Clinical Diagnosis
III. Developmental Anomalies and General Pathology

NORMAN L. GARLICK, B.S., D.V.M.

Tacoma, Washington

Part I of this series of papers which appeared in the April, 1954, issue of the American Journal of Veterinary Research *covered briefly the essentials of histology and embryology of the teeth of cattle. The research background for the material herein presented was given, along with a bibliography for suggested further study.*

Part II of this series which appeared in the July, 1954, issue of the same magazine covered the gross anatomy of the teeth and their response to environment and attrition and included a table for approximating the age of mature cattle by the appearance of the incisor teeth.

Part III here presented deals with developmental anomalies, injuries, and general pathology of the teeth of the ox, and a tabulation of these conditions as found in animals slaughtered for beef.

Developmental Anomalies

Incisor Teeth Not Formed.—Occasionally, tooth buds fail to form or to develop. In the permanent incisor group, the fourth pair is the set most often absent. The second pair of incisors has been observed to be absent; in 1 animal, the temporary second incisors were retained in their place. In the temporary dentition the fourth pair of incisors has been observed to be absent occasionally.

Cheek Teeth Not Formed.—In a small percentage of animals, the permanent tooth bud of the first mandibular premolar on one or both sides fails to form. Its temporary predecessor may or may not have been present. The permanent element never forms if the corresponding temporary tooth was not present. The defect is more common in the permanent than in the deciduous dentition and is hereditary in nature. The fourth upper cheek tooth has been observed to be absent, the gingiva presenting no scar which would indicate loss of the tooth. It is concluded, therefore, that other permanent cheek teeth may fail to develop in rare instances (fig. 1).

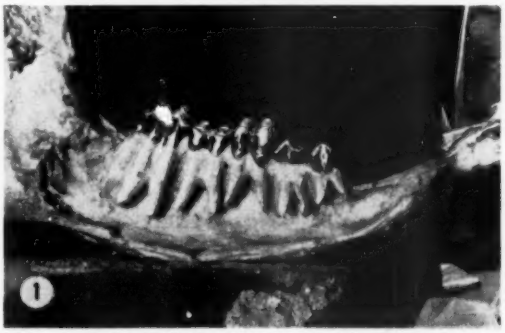


Fig. 1—Congenital absence of the first permanent mandibular premolar in a 3-year-old Hereford steer.

Supernumerary Teeth.—In only one instance was a supernumerary tooth observed. That occurred in the permanent incisor group, giving a total of nine instead of the usual eight. Colyer [1] reports a specimen with 24 incisor teeth.

Crowded Incisor Teeth.—Underdevelopment or retarded development of the anterior portion of the mandible results in the crowding and partial rotation of a number of incisor teeth. This defect is common in the permanent dentition where it has a tendency to gradually correct itself as the jaw develops more fully and as the wider portions of the crowns wear back. Crowding of the incisor teeth is rare in the deciduous dentition. In regard to crowding of teeth, it would be well to recall that at the time of eruption of the first permanent pair of incisors, there is often insufficient room for them. As a result, they are commonly seen to overlap at the medial border, being directed upward and inward. Jaw development soon corrects the physiological malposition in most cases.

Crowded Cheek Teeth.—Crowding of the cheek teeth in the ox has been observed but is rare. In a great number of animals, the opposite effect is noted. A space between

The author acknowledges the financial aid of the Aluminum Company of America whose research grant covered the major portion of the expense incurred in this investigation. However, the statements and conclusions expressed in this article are those of the author alone.

The author is engaged in consultation and research work in Tacoma, Wash.

the third and fourth cheek teeth is often seen. The space may contain a part of the posterior portion of the deciduous third premolar which becomes wedged in firmly during eruption of the permanent dentition. It may persist for many years. When crowding of the teeth does occur, it usually results in partial rotation of a tooth to relieve the pressure.

TABLE 1 — Source and Age of 7,480 Cattle Slaughtered for Beef and Used in This Study

Location of abattoirs	No. anim.
Los Angeles, Calif.	3,166
Phoenix, Ariz.	155
E. St. Louis, Ill.	4,159
Animal age groups	**No. anim.**
Young anim. without permanent incisor teeth erupted	2,302
Young anim. in period of eruption of permanent incisor teeth	3,375
Mature animals*	1,803

* Of the mature animals, 707 were estimated to be over 8 years of age based on the table of age approximation given in Part II of this series.

Rotation of Incisor Teeth.—Occasionally, the permanent incisor teeth will be observed to be rotated 90 or 180 degrees in the alveolus, though no evidence of crowding or other cause is apparent. In all such cases observed, the defect was noted in both individual teeth in any given pair.

Rotation of Cheek Teeth.—Rotation of cheek teeth has been observed only in the case of the first permanent mandibular premolar. The rotation is approximately 90 degrees. No observable cause could be determined. The defect has not been observed in the deciduous dentition.

Malposed Incisor Teeth.—Permanent incisor teeth may erupt out of the dental arch. Of course, these are nonfunctional and often interfere with prehension of food. Their deciduous predecessors are usually retained for a variable period of time.

Malposed Cheek Teeth.—Cheek teeth have been observed to erupt out of the dental arch and at odd angles. Most of the variations occur in the premolar group of the permanent dentition. As a result, the masticatory value of the tooth in the abnormal position, as well as a portion of that of its opponent is lost.

Jaw Deformities.—Brachygnathia, prognathia, and rotation of the anterior portion of the mandible have been observed. The extent of the defect will determine the relative ineffectiveness of the incisor teeth.

Irregular and Whorled Enamel.—The teeth occasionally present enamel prisms which deviate from their normal direction. These may be grossly apparent by resulting irregularities of the surfaces of the teeth. The normal corrugated, vertically grooved pattern may be lost to one of scalloped contours resembling the surface of an orange (fig. 2). Examination of ground sections of the tooth will demonstrate the abnormal prism direction. This defect apparently does not interfere with normal use or lifetime of teeth so affected. It has been observed in the permanent dentition only, and is always present in the same portion of both individuals in any given pair. Whorled and irregular enamel on the cheek teeth has been observed in the abattoir but would not be observed easily in the living subject. Similar defects have been described in the human being.[2]

Deep Vertical Folds in the Surface of Incisor Teeth.—Sometimes the dentinoenamel junction becomes infolded prior to the formation of enamel and dentine. The result is a single deep vertical sulcus on the labial surface of both individual teeth in any given pair (fig. 3). This does not interfere with the use or average lifetime of the tooth. Pigments tend to collect in the depths of the groove, and the enamel cuticle persists for a longer time in the deeper portions of the depression.

Accessory Cusps on Incisor Teeth.—The labial surface of the permanent incisor teeth may occasionally present an accessory cusp, particularly on the fourth pair (fig. 4). The cusp is present bilaterally and may be of considerable size. This is a developmental oddity which does not interfere with the usefulness of the teeth involved. Because of its prominent position, the cusp is soon lost to the effect of normal attrition and the tooth may later appear normal. The tendency for the fourth incisors to develop an accessory cusp accounts for the fact that the labial surface of that pair is usually more irregular than that of the other three (fig. 5).

Macrodontia Involving Incisor Teeth.—The development of abnormally large incisor teeth in the ox has been observed in the permanent dentition. It involves both individuals in any given pair. The normal form may give way to a three-sided or four-

sided structure. In case three sides form, there is a more deeply concave single lingual surface and double labial surfaces which merge to form a triangular-shaped tooth when viewed from the incisal margin (fig. 6). Deep vertical sulci form in the double labial surfaces, due to infolding. The enamel at the tip may not lie in perfect apposition but may be separated by a shallow infundibulum lined with dentine. The roots are enlarged and present three vertical sulci which terminate at the cemento-enamel junction at each corner of the triangular crown. The enamel does not cover the dentine at the lower part of the anterior angle of the tooth and, as a result, the gingival attachment remains higher at that point. The tendency for the gingival attachment to follow the contour of the cemento-enamel junction is remarkable.

When macrodontia is accompanied by formation of an incisor tooth with four surfaces instead of the normal two, two of the surfaces resemble the normal labial surface and face labially and lingually. The other two surfaces resemble the normal lingual surface, are deeply concave, and replace the medial and lateral margins of the crown

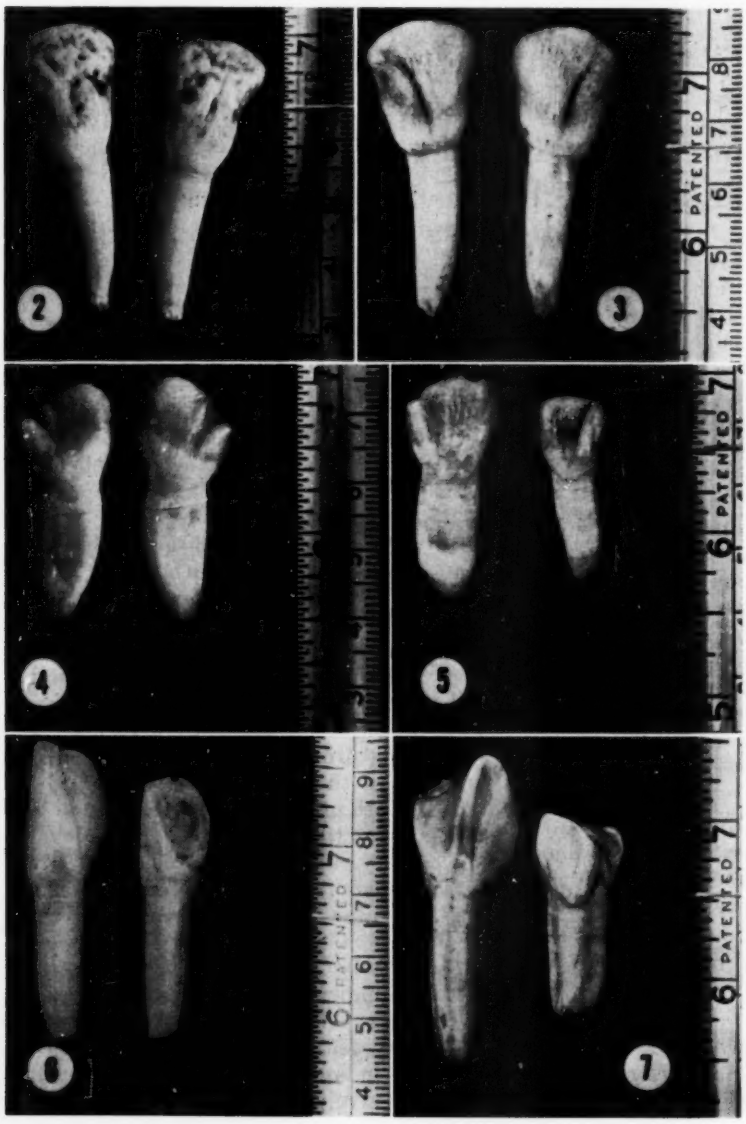


Fig. 2 — Irregular enamel accompanied by areas of enamel hypoplasia which do not clearly follow the incremental pattern. Surface pigments have lodged in the depressions. The remaining enamel is normal in luster and hardness.

Fig. 3 — Abnormal infolding of the dentino-enamel junction early in formation of these teeth resulted in the deep vertical sulci seen here on the surface of the enamel.

Fig. 4 — Accessory cusps on the fourth pair of permanent incisor teeth.

Fig. 5 — Irregular formations of fourth incisor teeth from 2 mature animals.

Fig. 6—Three-sided incisor teeth from 2 different animals, from anterolateral aspect (left) and from posteromedial aspect (right).

Fig. 7—Four-sided incisor teeth from 2 different animals, from a 4-year-old (left) and from a 7-year-old (right). The left-hand portion of the tip of the larger tooth was fractured during slaughter.

(fig. 7). The root is enlarged and presents four vertical sulci which terminate at the cemento-enamel junction at each angle of the crown. The defective formation is bilateral.

Macrodontia Involving Cheek Teeth.—One case of macrodontia has been observed in a permanent maxillary premolar. It was a simple enlargement, with no compound dental structures. It was unilateral.

Portions of Mandibular Molars Developing as Individual Teeth.—The anterior and posterior halves of the fifth mandibular cheek tooth have been observed to develop as two individual teeth with no fusion between. The defect was bilateral. The two teeth were slightly smaller than a single normal tooth. The same phenomenon has been observed in the sixth mandibular cheek tooth, except that it developed as three separate units.

TRAUMATIC INJURIES OF THE TEETH

Fractured Incisor Teeth.—Transverse fractures have been observed in both permanent and temporary incisor teeth. They do not occur very often because of the relatively loose alveolar implantation.

Fractured Cheek Teeth.—Fractures of the cheek teeth in the ox are fairly common, occurring more often in the mandibular group. Most are probably due to the attempted mastication of some hard object, and usually split off the lateral cusps or margins of the mandibular teeth. Any type of fracture can occur, however. The fracture runs downward and outward and usually does not involve the root. Fractured surfaces soon wear smooth, but a close examination will serve to differentiate them from hypoplastic deformities, since the fracture does not follow the incremental pattern of development, while the latter does.

Depression of Incisor Teeth.—Occasionally, an incisor tooth is driven forcefully downward and backward into the alveolus. This may result in a fracture of the alveolar bone allowing the apex of the root to lodge in the connective tissue beneath the oral mucosa on the labial margin of the dental arch. Strangely, though the trauma is apparently great, the teeth retain or regain their pulpal circulation. The alveolar fracture is repaired and the tooth remains in its depressed position.

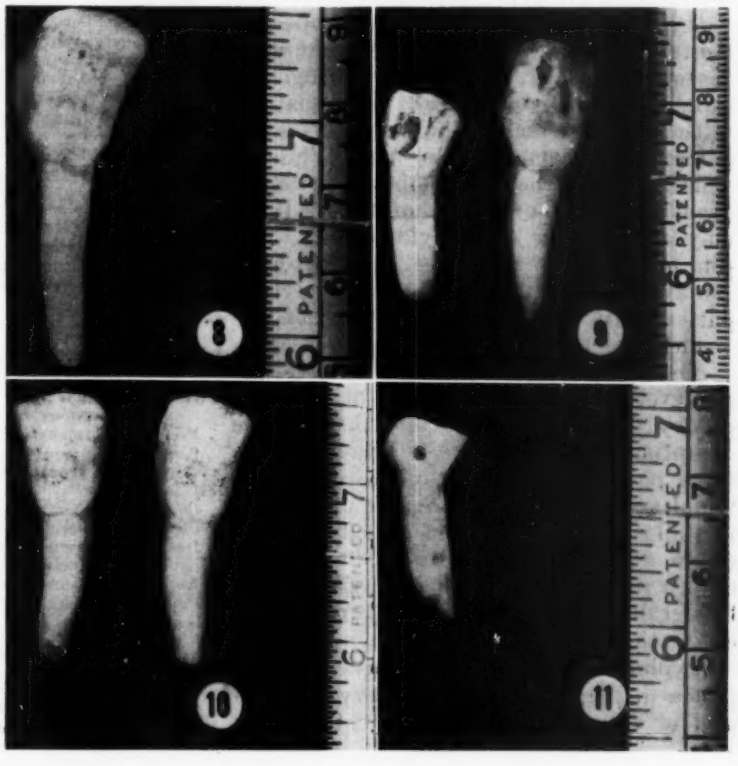


Fig. 8—Two forms of hypoplasia in the same tooth. Near the tip is a rather wide transverse groove which reaches the dentine in its deeper distal portions. Near the lower third of the enamel surface can be seen a transverse row of pits.

Fig. 9—Hypoplasia of the enamel in the form of a single large pit on the left, and a horizontal row of large pits on the right.

Fig. 10—Chalky incisor teeth showing excessive attrition and erosion of the enamel. This pair of teeth had been in wear about seven months. The rest of the teeth in this animal were of normal quality.

Fig. 11—A deciduous incisor tooth removed shortly before time of shedding. A well-defined enamel fleck can be seen in the center of the crown.

Miscellaneous Traumatic Injuries.—Though other traumatic influences may cause many and varied injuries to the teeth, they need not be described here, as they are usually grossly evident and easily recognized.

General Pathology

Retained Temporary Teeth.—In addition to the causes of retention of deciduous elements already brought out, others should be mentioned. Practically all serious systemic illnesses tend to retard dental development and foster retention of the deciduous dentition. Permanent teeth are delayed for variable periods of time. Generalized malnutrition, as well as deficiencies of individual nutritive factors, may have the same results although, in the latter case, the teeth may erupt on schedule but be of inferior structure. A great deal of research has been done on animals to determine the effects of these factors. The bibliography accompanying this article (given with Part I) will give the source and results of most of this work, which is too voluminous to report here. It does help to give further insight into the probable causes of the defects which are being described here.

Hypoplasia.—Hypoplasia as applied to dental elements refers to underdevelopment of tooth structure. It can be recognized grossly in the living subject by variations in the enamel which follow the incremental pattern of enamel apposition. While it usually takes the form of a horizontal groove across the surface of the tooth, it may also appear as individual pits. The pits may or may not be arranged in horizontal pattern but never take a vertical pattern. In uncomplicated hypoplasia the enamel which does form is of good quality.

Hypoplasia of Temporary Teeth.—Underdevelopment of the enamel of the temporary teeth is seldom observed, with one exception. A hypoplastic pit occurs on the lower portion near the medial border of the labial surface of the third pair of incisors in well over 50 per cent of all calves. It is of no detriment to the individual, being only a developmental oddity. It is always bilateral and does not extend through the entire thickness of the enamel.

Hypoplasia of Permanent Teeth.—Hypoplasia in permanent teeth is very common and of universal geographic distribution. It is due to a great many different systemic disturbances, yet gives a constantly similar pattern. If at any time during the appositional phase of enamel formation a systemic disturbance occurs which is of a sufficiently serious nature that it will interfere with enamel prism formation, the active ameloblasts discontinue their normal function and become modified. They can not resume

TABLE 2—Dental Developmental Anomalies, Injuries, and Pathological Conditions Encountered in 7,480 Cattle Slaughtered for Beef

Abnormal condition	No. anim.*
Enamel flecks in permanent incisor teeth	1,026
Enamel flecks in temporary incisor teeth	Not recorded
Simple hypoplasia in permanent incisor teeth	221
Simple hypoplasia in temporary incisor teeth	Not recorded
Dental caries in permanent teeth	343
Dental caries in temporary teeth	Not recorded
Dental fluorosis in permanent teeth	80
First permanent mandibular premolar not formed	72
First temporary mandibular premolar not formed	1
Permanent cheek teeth, other than the first mandibular premolar, not formed, or missing without evidence of prior existence	23
Poor quality permanent cheek teeth	43
Poor quality temporary cheek teeth	1
Rotated permanent incisor teeth, degree of rotation 90 or more	18
Rotated permanent cheek teeth	6
Accessory cusps on permanent incisor teeth	11
Abnormal crowding of permanent incisor teeth	10
Macrodontia of permanent incisor teeth, including those with compound structure	9
Permanent incisor teeth not formed	6
Temporary incisor teeth not formed	1
Crowded permanent cheek teeth	1
Jaw deformities	4
Supernumerary permanent incisor teeth	1
Irregular and whorled enamel on permanent incisor teeth	2
Retained temporary incisor teeth	3
Malposed permanent incisor teeth	3
Malposed permanent molar teeth	1
Malformations of permanent cheek teeth	9
Deep vertical sulcus on labial surface of permanent incisor teeth	1
Portion of temporary premolar retained in alveolus	1
Hypertrophy of enamel on permanent incisor teeth	1
Fifth mandibular cheek teeth formed in two portions	1
Sixth mandibular cheek teeth formed in three portions	1
Fractured permanent teeth	10
Fractured temporary teeth	Not recorded
Traumatic depression of permanent incisor teeth	2
Chalky permanent incisor teeth	13
Chalky tip of permanent incisor tooth	1
Deeply stained enamel in a fractured permanent incisor tooth	1
Abscessed permanent teeth	10
Portion of permanent cheek tooth of poor quality	6

* From the standpoint of pathology, the animals represented in this table can not be considered a cross section of average bovine animals in the United States. In the average mature animal, the incidence of abnormalities is much higher than is apparent here.

their work when the disturbance is over. Those which have not yet started active deposition of enamel prisms are not affected and can begin to function at their pre-

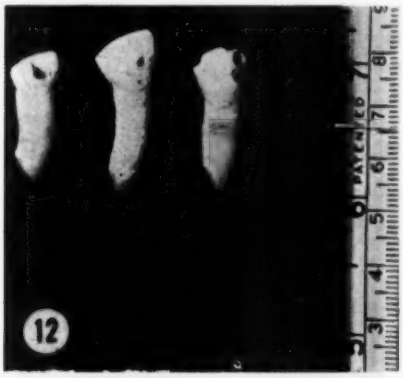


Fig. 12—Enamel flecks on incisor teeth from 3 different animals. In addition, the tooth on the right presents an area of hypoplasia in the pit below the deeply pigmented enamel fleck. The pit is outlined by surface discoloration.

scribed time as if nothing had happened. It seems, also, that the ameloblast has been given just so long to get its work done and, if not finished, forms the cuticle anyway, leaving a horizontal groove that corresponds exactly with the duration of the disturbance. The groove blends with the normal enamel surface at the point nearest the growth center and is deep distally (fig. 8). The vertical width of the groove depends entirely on the severity and duration of the disturbance. When apposition of enamel begins again, the first prisms to form are directed in a more vertical direction and overlie the area of malformation, partially covering it.

If the disturbance is of a minor nature, only a portion of the ameloblasts in any given area may be inhibited, resulting in the pitted appearance already mentioned (fig. 9). The pits themselves are arranged in a horizontal pattern if any pattern exists at all. Because of the horizontal appositional formation of enamel, the pattern can never be vertical.

Hypoplastic defects are easily seen on incisor teeth but, because of dental calculus deposits, are seldom seen on cheek teeth unless they occur on the exposed portions. Knowing the time of development of each tooth, one can closely approximate the age at which the disturbance occurred even though many years may have passed. The defect, once formed, can never be repaired. The approximate active appositional stage of enamel formation in any given incisor tooth has been observed to begin about one year prior to eruption and to end when eruption takes place.

DISTURBANCES IN CALCIFICATION

Faulty calcification of the teeth may be of either or both of two types. The first, hypocalcification, is characterized by the presence of inorganic salts probably normal in chemical composition but deficient in quantity. The deficit may be in all portions of the tooth or involve only one tissue, such as the interprismatic substance of the enamel. The second, inferior inorganic salt formation, may present a nearly normal amount of calcium compounds but of inferior structural hardness. The inorganic salts of the teeth are not of constant chemical composition but vary as a result of various influences. Observed conditions in which disturbed calcification plays a part are many. Research has indicated that imbalances of inorganic elements and/or vitamin D result in disturbances of dental calcification, but the degree of the involvement can not be prognosticated with accuracy. Endocrine disturbances, particularly of the parathyroid glands, have been incriminated. Diseases, particularly those of the skeleton, have received considerable attention but, again, the dental defects can not be prognosticated with any degree of accuracy. Recent severe dietary deficiency

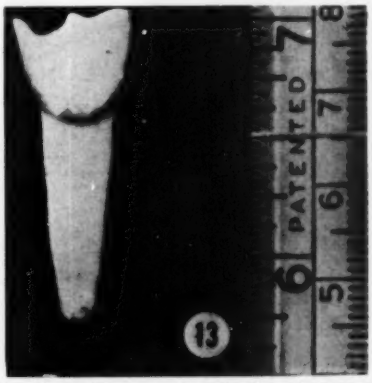


Fig. 13—The large notch in the left-hand portion of the enamel margin of this tooth was caused by the wearing away of an enamel fleck. The last remnants of the defect were found at the deepest margin of the notch.

studies in sheep have produced constant pathological patterns.[5]

OBSERVED ABNORMALITIES WHICH REFLECT DISTURBED MATURATION OF ENAMEL

Poor Quality Teeth.—Teeth of inferior quality are sometimes seen, both in permanent and more rarely in temporary dentition, involving all of the teeth to an equal degree. They may appear to be normal though perhaps of slightly less translucency but tend to wear back at a faster than usual rate.

Brown Teeth.—Teeth of inferior quality may present a brown pigmentation which may penetrate the entire structure. Temporary teeth, particularly, present the latter condition. Brown teeth are of universal geographic distribution. The pigment sometimes varies slightly in intensity in various parts of the tooth, giving a somewhat mottled appearance. The condition must not be confused with dental fluorosis, since the latter has not been observed by the author to affect the deciduous teeth of the ox. The occurrence of brown teeth in the temporary dentition is of no greater incidence in endemic fluorosis areas than elsewhere. Inheritance may play a part in the occurrence of brown teeth.[3]

Incomplete Enamel Maturation.—Cheek teeth are sometimes seen which are yellow-brown in color, the enamel being soft, opaque, and lusterless. The condition is usually bilateral in any given pair of teeth. Sometimes only half of the tooth will be involved, either the posterior or anterior portion in the case of the cheek teeth. The shape of the tooth is normal and complete. Organic composition is apparently normal.

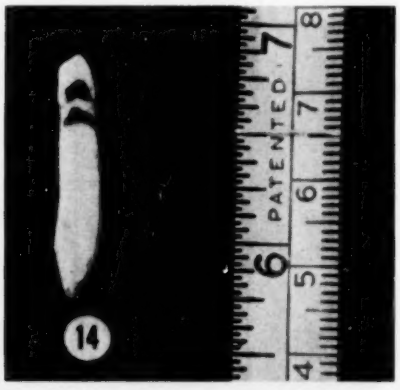


Fig. 14—Caries of the dentine of the root of a deciduous incisor tooth. Two lesions can be seen. The tooth has lost its crown to processes of attrition and abrasion, but root resorption has not yet begun.

Fig. 15—An unusual case of caries of primary dentine. The root deformity resulted in an abnormal contact of the crown of this incisor tooth with its neighbor. Where contact existed, the enamel wore away exposing primary dentine. The carious process developed, protected by the proximity of the neighboring tooth. The spot at the center of the tooth is a well-defined enamel fleck.

The condition is universal in geographic distribution and more often involves permanent than temporary teeth. The cause is probably a defective tooth germ which does not have the ability to foster complete maturation. The dentine is of inferior quality, also, as is evident by the fact that the entire structure is susceptible to the action of putrefactive bacteria which often cause decomposition and elimination of the teeth so affected. There is a severe abscess formation in the structures surrounding a decomposing tooth. The odor is distinctive and prominent.

Chalky Teeth.—Teeth presenting an opaque, lusterless, chalky appearance are commonly seen. They may be described in two groups, separated as to probable cause. The first, chalky teeth due to systemic disturbances other than dietary, may involve the entire enamel of the tooth or only a portion of the visible crown. The defect is in the enamel only and is bilateral. The cause is a systemic disturbance which interferes with enamel maturation. It follows the pattern of secondary calcification, the entire depth of the enamel being involved. The interprismatic substance is primarily

faulty. The tips of the enamel prisms break off and crumble away at an increased rate. The condition is universal in geographic distribution. Temporary teeth have not been observed to be affected.

The second, chalky teeth due to dietary deficiencies and imbalances, may result in the enamel of the entire permanent dentition being soft, opaque, and chalky. This condition is sometimes limited geographically. It can be favorably influenced by control of known dietary discrepancies. Not all animals are equally susceptible, all degrees of the condition being manifest in various individuals subject to the same environment. The teeth have a tendency to absorb pigments on the surface producing a dull yellow color (fig. 10).

Opaque Enamel Spots—"Enamel Flecks"

The occurrence of opaque spots in the enamel of both the permanent and temporary teeth is so universally common that a separate section of this paper is needed to indicate its relative importance. Approximately 65 per cent of all mature "full-mouthed" cattle will show the defect on at least one pair of incisor teeth. The set most often involved is the third. The defect is usually bilateral and may be multiple on any given tooth. It is universal in geographic distribution. It is hereditary in nature, having a tendency to run in families.

Enamel flecks are seen on the labial surface of the incisor teeth. The spots follow no constant pattern but are usually present in the same location in the two individuals of any affected pair though they often appear unilaterally. The color varies from an opaque white through all shades of yellow and brown to a deep black. The pigmentation becomes more prominent as age advances. The margins of the defective areas are clear, distinct, and abrupt, there being no blending with the surrounding normal enamel. However, the pigmentation, when present, is of a darker color in the center of the area than at the periphery (fig. 11 and 12).

The probable fundamental pathology is an absence of, or hypocalcification of, the interprismatic substance of the enamel.[4] The prisms are normal but have inadequate support. That the areas are extremely soft in comparison to normal enamel is evident when they come into wear. In the normal process of attrition, the teeth may be worn until an enamel fleck is incorporated into the incisal margin. Immediately a notch develops in the edge of the enamel and the defective area is soon completely worn away (fig. 13). Increased erosion of the defective area is evident, also, after the enamel cuticle has worn away, the surface receding faster than that of the surrounding normal enamel. After the enamel cuticle is gone, the area seems to be rough when scraped, due to protrusion of the exposed tips of the enamel prisms. Enamel flecks are subject to caries.

Dental Caries

Dental caries of the teeth of the ox is common. The condition increases in incidence as age advances. It is universal in geographic occurrence and is found in both temporary and permanent dentitions. It has been observed to occur in both dentine and enamel.

Fig. 16—Caries of secondary dentine.
Fig. 17—Caries in enamel flecks in incisor teeth. The second tooth from the left presents an area of hypoplasia of the enamel at the base of the crown.

Caries of the Dentine.—Caries have occasionally been observed to attack primary dentine (fig. 14 and 15). Secondary dentine is very commonly affected, the process beginning at any time after the secondary

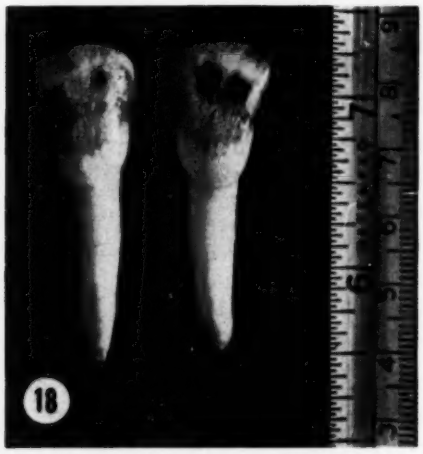


Fig. 18—Three forms of pathological conditions, all involving the same portion of a set of incisor teeth from a 2-year-old animal. A wide area of hypoplasia involves most of the upper half of the crown. Hypocalcification of the interprismatic substance in the same portion of the tooth is outlined by opaque enamel. Four carious lesions involve the hypocalcified areas.

dentine is exposed in the normal process of attrition (fig. 16). At first the affected tissue may appear orange but is usually black. As the process continues, the decaying tissue is lost and the tooth is gradually hollowed out along the pattern of the original pulp cavity. Caries may reach the apex of the root and periodontal abscesses have been observed as a result. The deposition of secondary dentine is accelerated by an attack of caries. The faster it is deposited, the more inferior the structure and the more susceptible it is to the progression of the process.

Caries of Enamel.—Caries has not been observed to attack normally formed enamel. It seems to occur only in the defective areas which have been previously described under the heading "enamel flecks" (fig. 17 and 18). It does not spread to underlying dentine nor to surrounding normal enamel. When the enamel fleck has entirely rotted away and the decayed tissue lost, the area appears to be a clean enamel pit which extends through the entire thickness of the enamel, not following the incremental pattern. Caries of the enamel is self-limiting in the ox. The depths of the pit may retain a black pigmentation for an indeterminate time. It is probable (and it has been observed occasionally) that many enamel pits seen in older animals were at one time enamel flecks, the defective tissue being lost to caries and erosion, leaving an apparent hypoplastic defect which does not follow the appositional pattern of enamel development.

Teeth affected by fluorides have been observed to demonstrate decreased incidence of caries.

SUMMARY

Specific dental developmental anomalies, common injuries of the teeth, and a number of dental diseases of the ox encountered in clinical investigations as well as research have been discussed. Special emphasis has been placed upon dental caries, enamel flecks, disturbed calcification, and hypoplasia. A tabulation of dental anomalies, injuries, and pathological conditions as found in animals slaughtered in abattoirs in various sections of the United States has been presented. Limited reference has been made to dental fluorosis which will be discussed in detail in Part IV of this series of papers to follow.

References

[1] Colyer, F.: Variations and Diseases of the Teeth of Animals. John Bale, Sons, and Danielsson, Ltd., London, (1936): 76.

[2] Thoma, K. H.: Oral Pathology. 3rd ed. C. V. Mosby Co., St. Louis, (1950): 286.

[3] *Ibid.*: 267-271.

[4] *Ibid.*: 271.

[5] Franklin, M. C.: The Influence of Diet on Dental Development in the Sheep. Commonwealth Scient. and Indust. Res. Organ., Bull. 252, Melbourne, Australia, 1950.

The pH Values, Carbon Dioxide Content, and the Levels of Sodium, Potassium, Calcium, Chloride, and Inorganic Phosphorus in the Blood Serum of Normal Cattle

B. J. McSHERRY, D.V.M., and I. GRINYER

Guelph, Ontario, Canada

THERE HAS been in the past twenty years an increasing interest in the chemical anatomy of man and animals, and changes from normal are reported in many pathological conditions. A knowledge of the physiology and chemical pathology of body fluids is essential in order that these changes can be recorded and evaluated.

Excellent references to recent advances made in this field in human medicine are contained in books by Gamble,[10] Bland,[3] Cantarow and Trumper,[4] Moyer,[15] and Harrison.[11] Readers are referred to these textbooks for the basic outlines of physiology and chemical anatomy on this subject. A review of the veterinary literature revealed numerous references to some of the normal values in cattle.[1,2,5,6,9,13,14,17,18,20,22,23]

The present paper presents data from a study of some of the constituents and properties of normal cattle serums that are likely to show changes in electrolytic upsets.

MATERIALS AND METHODS

The investigation was conducted on a herd of Holstein-Friesian cattle. Observations were made over a period of one year on cattle at pasture as well as during the feeding period in the stable. The health of these animals was considered to be above average; 126 animals were used in the study, 86 of which were 2 years of age and over, 20 were calves from 4 to 10 months of age, and 20 were calves from 4 days to 10 weeks of age. The mature animals were at various stages of pregnancy and lactation.

The serum was collected by using a 5-in. length of soft gum rubber tubing similar to that used with red and white cell pipettes. The rubber tubing was fitted with needle adapters at both ends. A 16-gauge, 2½-in. needle was attached to one adapter and a 16-gauge, 4½-in. needle to the other. Test tubes, 10.0 by 1.5 cm., were filled to a depth of 2.0 cm. with mineral oil. All the equipment was thoroughly cleaned and rinsed with distilled water. The 2½-in. needle was inserted into the jugular vein and blood was allowed to flow from the other needle for about thirty seconds to evacuate all the air from the tubing. The rubber tube was then pinched off and the 4½-in. needle was inserted into the test tube below the surface of the mineral oil. The blood flow was then allowed to continue, being controlled so that the 2.0-cm. layer of mineral oil covered the blood at all times. Four, 9.0-cc. samples were collected in this manner from each of the animals.

The samples were allowed to clot at room temperature for a period of approximately two hours and then were centrifuged in the original tubes. The carbon dioxide content and pH values were determined immediately after centrifugation and, at this time, the remainder of the serum was pipetted and transferred under oil to fresh test tubes. These were sealed with rubber stoppers and stored in a refrigerator for three to four days at which time sodium, potassium, calcium, chloride, and phosphorus determinations were made.

The amount of carbon dioxide was determined by the method of Peters and Van Slyke.[16] The pH values were obtained colorimetrically, following the method of Hastings and Sendroy,[12] and using bicolor standards prepared in this laboratory. Se-

From the Ontario Veterinary College, Guelph, Ontario. Mr. Grinyer is an associate of the Institute of Medical Laboratory Technology, England. Dr. McSherry is assistant professor.

TABLE 1—Mean and Standard Deviation of Some Inorganic Constituents and pH Values in the Serums of Normal Dairy Cattle

Group		Milliequivalents of constituents per liter of serum					Mg. per 100 cc.	
Age	No. of animals	Na^+	K^+	Ca^{++}	HCO_3^-	Cl^-	HPO_4^-	pH
2 to 13 years	86	142.0 ±5.0	4.85 ±0.47	5.42 ±0.34	29.47 ±1.96	103.7 ±3.5	4.5 ±0.94	7.42 ±0.057
4 to 10 months	20	143.1 ±6.6	5.12 ±0.45	5.58 ±0.4	29.34 ±1.92	102.43 ±3.16	7.8 ±1.09	7.4 ±0.14
4 days to 10 weeks	20	142.05 ±4.0	5.25 ±0.54	5.08 ±0.22	31.03 ±1.9	103.0 ±2.49	7.6 ±1.1	7.44 ±0.14

rum chlorides were determined on 0.2 cc. of serum by the method of Schales and Schales.[19] Fiske and Subbarrow's method[8] was used for phosphorus levels. Sodium, potassium, and calcium were determined by flame photometry, using a synthetic serum containing 144.0 mEq. sodium, 4.8 mEq. potassium, and 5.0 mEq. of calcium per liter as an external standard. The Beckman Model DU Spectroflame photometer was used.

Results

Table 1 is self-explanatory and condenses the information realized during this study.

The inorganic serum constituents are within the ranges expressed by previous workers.[2, 6, 9, 14, 17, 18, 20] The pH ranges agree with those given by Dukes,[7] but are higher than the results obtained by Craige, Johnson, Blackburn, and Coffin.[5] A comparison of the $HPO_4^=$ levels showed that there was a highly significant difference between the animals 4 days to 10 months of age and those 2 to 13 years of age ($t = 4.6$; $df = 104$; $P < 0.01$). These correspond to the values reported by Vanlandingham, Henderson, and Bowling.[21]

Conclusions

The pH values, carbon dioxide content, and the level of sodium, potassium, calcium, chloride, and inorganic phosphorus of serum from normal cattle were determined in 126 dairy cattle. The results are recorded and statistically analyzed in table 1 and show that, between the age groups tested, the only significant difference was in the $HPO_4^=$ levels.

References

[1] Anderson, A. K., Gayley, H., and Pratt, A.: Studies on the Chemical Composition of Bovine Blood. J. Dai. Sci., *13*, (1930): 336-348.

[2] Bergmann, E. N., Sellers, A. F.: Studies on Intravenous Administration of Calcium, Potassium, and Magnesium to Dairy Calves. Am. J. Vet. Res., *14*, (1953): 520-529.

[3] Bland, J. H.: The Clinical Use of Fluid and Electrolyte. W. B. Saunders Co., Philadelphia, 1952.

[4] Cantarow, A., and Trumper, M.: Clinical Biochemistry. 4th ed. W. B. Saunders Co., Philadelphia, 1949.

[5] Craige, A. H., Johnson, R. B., Blackburn, E. G., and Coffin, I. M.: Biochemical Studies on Three Cows at Parturition Including One Case of Milk Fever. J.A.V.M.A., *114*, (1949): 136-140.

[6] Dennis, J., and Harbaugh, F. G.: The Carbon Dioxide Content of the Blood of Dairy Cattle. Am. J. Vet. Res., *7*, (1946): 37-40.

[7] Dukes, H. A.: The Physiology of Domestic Animals. 6th ed. Comstock Publishing Co., Inc., Ithaca, N. Y., (1947): 17.

[8] Fiske, C. H., and Subbarow, Y.: The Colorimetric Determination of Phosphorus. J. Biol. Chem., *66*, (1925): 375.

[9] Fain, P., Dennis, J., and Harbaugh, F.: The Effect of Added Manganese in Feed on Various Mineral Components of Cattle Blood. Am. J. Vet. Res., *13*, (1952): 348-350.

[10] Gamble, J. L.: Chemical Anatomy, Physiology, and Pathology of Extracellular Fluid. Cambridge-Harvard University Press, 1947.

[11] Harrison, T. R.: Principles of Internal Medicine. The Blakiston Co., New York, (1951): 293-305.

[12] Hastings, A. B., and Send.oy, J.: The Colorimetric Determination of Blood pH at Body Temperature Without Buffer Standards. J. Biol. Chem., *61*, (1924): 695-710.

[13] Houghin, O. B., Graham, W. R., Peterson, V. E., and Turner, C. W.: The Chemical Composition of the Blood of the Dairy Goat. J. Dai. Sci., *22*, (1939): 241-249.

[14] Lengemann, F. W., Aines, P. O., and Smith, S. E.: The Normal Chloride Concentration of Blood Plasma, Milk, and Urine of Dairy Cattle. Cornell Vet., *42*, (1952): 28-35.

[15] Moyer, C. A.: Fluid Balance—A Clinical Manual. The Year Book Publishers, Chicago, 1952.

[16] Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry. Vol. 2, The Williams and Wilkins Co., Baltimore, 1932.

[17] Reihart, O. F.: Chemical Studies of the Blood of Normal Cows. J.A.V.M.A., *94*, (1939): 33-34.

[18] Sampson, J., and Hayden, C. E.: The Acid Base Balance in Cows and Ewes During and After Pregnancy with Special Reference to Milk Fever and Acetonemia. J.A.V.M.A., *86*, (1935): 13-22.

[19] Schales, O., and Schales, S. S.: A Simple and Accurate Method for the Determination of Chloride in Biological Fluids. J. Biol. Chem., *140*, (1941): 879-884.

[20] Sellers, A. F., Roepke, M. H.: Studies of Electrolytes in Body Fluids of Dairy Cattle. Am. J. Vet. Res., *12*, (1951): 292-295.

[21] Vanlandingham, A. H., Henderson, H. O., Bowling, G. A.: Studies on the Chemical Composition of the Blood of Dairy Cattle. J. Dai. Sci., *25*, (1942): 548.

[22] Ward, G. M.: The Relation of Prepartal and Postpartal Mineral Balances to the Occurrence of Parturient Paresis in Dairy Cows. J. Dai. Sci., *35*, (1952): 587-594.

[23] Ward, G. M.: Blood Levels of Some Inorganic Constituents in Normal Parturient Cows and Cows with Parturient Paresis. J. Dai. Sci., *36*, (1953): 39-44.

An Evaluation of the Complement-Fixation Test for Anaplasmosis

K. E. PRICE, Ph.D.; W. E. BROCK, D.V.M.; J. G. MILLER, D.V.M., V.S., M.S.

College Park, Maryland; Pawhuska, Oklahoma; Baton Rouge, Louisiana

ANIMALS (Bovidae) that recover from an attack of anaplasmosis generally become life-long carriers of the disease and only rarely can this carrier condition be revealed by examination of blood smears. This difficulty in diagnosing carrier animals has forced investigators to turn to serological diagnosis.

LITERATURE CITED

Rees and Mohler[1] demonstrated the presence of complement-fixing antibodies using as an antigen a saline extraction of ticks which had engorged themselves on the blood of infected animals. As only small quantities of antigen could be produced by this method, it was discarded.

Boynton and Woods[2] showed that serums of newly recovered carriers would frequently give a cloudy precipitate in distilled water, whereas serums of anaplasmosis-free animals failed to cause any precipitation. These investigators indicated that this test was based upon the precipitation of euglobulin. Since the test is not specific for anaplasmosis and carriers of long standing give little reaction, this method has not received wide use.

In more recent studies Mohler, Eichhorn, and Rogers[3] reported a satisfactory complement-fixation test for bovine anaplasmosis. The preparation of antigen for this test was described by Mott and Gates.[4] In many cases, antigens prepared by this method were unsatisfactory due to anticomplementary activity or to a lack of antigenicity.

In 1952, Price, Poelma, and Faber[5] described an improved antigen preparation procedure. The improvements in antigen quality were brought about by removal of a great deal of extraneous material which resulted in a purer, more concentrated antigen. Practical application of the complement-fixation test employing antigen prepared by this method was found highly satisfactory. Its use in removing all carrier animals from three Maryland herds was described by Price, Poelma, Hastings, and Mitchell[6] in 1953.

From the Live Stock Sanitary Service, College Park, Md. (Price); Oklahoma Agricultural and Mechanical College, stationed at Veterinary Research Field Station, Pawhuska (Brock); Louisiana State University and Agricultural and Mechanical College, Baton Rouge (Miller).

This work was carried out under an agreement of cooperation between directors of the Agricultural Experiment Stations of Maryland, Oklahoma, and Louisiana and supported by funds from each of the stations.

As project leaders, the authors wish to express their appreciation to the personnel of their respective institutions for technical assistance.

The purpose of the present investigation was twofold. The first objective was to contribute additional data in regard to the efficacy of the complement-fixation test. This was accomplished through testing a large number of samples from animals with known histories.

The second objective was to obtain information which would solve some of the problems that arose in the course of complement-fixation work carried out by the authors at their various institutions. Among the most important of these problems were (1) the need for standards on which to base the interpretation of results; (2) the need for knowledge as to the relative accuracy of the spectrophotometric and visual method of reading the results of complement-fixation test; (3) information as to the uniformity of antigens prepared at the different institutions.

MATERIALS AND METHODS

A total of 278 individual blood serum samples were tested by each of the three investigators during the course of this experiment. The samples were obtained from animals whose history, as far as anaplasmosis was concerned, was well known. The negative or positive status of the animals was proved by injection of blood into a susceptible individual or by direct blood smears during acute infection. These serums were preserved by freezing. Satisfactory preservation was obtained in all cases with no observable loss in antibody titer.

The antigens utilized in this study were prepared at the various institutions according to the method outlined by Price *et al.*[5] in 1952. Miller employed several modifications which served to reduce the time required for preparation as well as to increase the yield of antigen. These modifications included the use of the Sharples centrifuge to concentrate the red blood cell matrices after the cells had been lysed in the initial stages of antigen preparation. Also, Miller used large volumes of blood for inoculation to increase the parasite yield of the blood to be used as a source of antigen.

The complement-fixation procedure was the following. Reagents of the hemolytic system were standardized spectrophotometrically. Standardization of the guinea pig complement was carried out by a 50 per cent end-point titration method based

on that developed by Kent *et al.*[7] and the optimal amboceptor concentration determined by a technique first employed by Kent.[8] Sheep red blood cells standardized spectrophotometrically at 2 per cent red blood cells in veronal-bicarbonate buffered salt solution.

All serums were inactivated at 56 C. for thirty minutes and diluted 1:10 with this solution which was used as the diluent throughout the test. The ingredients and proportions are:

sodium chloride	16.76 Gm.
sodium bicarbonate	0.50 Gm.
sodium 5,5 diethyl barbiturate	0.6 Gm.
5,5 diethyl barbituric acid	0.92 Gm.

Dissolve the acid in 500 cc. of hot distilled water; add to the solution of other salts and q.s. to 2,000 cc. with distilled water. The final pH of this solution will be 7.3 to 7.4.

Antigen titrations were carried out by preparing serial twofold dilutions of antigen 1:3 through 1:24 with the additional dilutions of 1:9 and 1:36. Three-tenths cubic centimeter of each of these dilutions was then added to three rows of tubes; the first row containing 0.3 cc. of a 1:10 dilution of a positive serum, the second 0.3 cc. of a 1:10 dilution of a negative serum, and the third 0.3 cc. of veronal-bicarbonate buffered salt solution. The dilution of antigen selected was one near the midpoint of the range between the highest dilution giving complete fixation in the positive serum and the highest dilution giving a reaction in the negative serum. A modification of this method was employed by Brock, who utilized both a strong and a weak positive serum in his titration procedure.

The antigen used at the Maryland laboratory was prepared in 1952. This antigen was reconstituted from the lyophilized state by adding 20 cc. of veronal-bicarbonate buffered salt solution to each 100 mg. of antigen. Each cubic centimeter of antigen suspension thus contained 5 mg. of antigen. When this antigen was titrated, the highest dilution of the positive serum giving a 4+ reaction was 1:24, while the 1:3 dilution was the only one of the negative serum series showing a reaction. A dilution of 1:12 was then adopted for routine testing of samples, making the final concentration of antigen 0.416 mg./cc. of suspension.

The antigen used at Oklahoma was prepared by the Oklahoma laboratory in April, 1953. This antigen was reconstituted from the lyophilized state by adding 30 cc. of veronal-bicarbonate buffered salt solution to each 100 mg. of antigen. Each cubic centimeter of antigen suspension thus contained 3.33 mg. of antigen. This antigen was titrated using a strong positive serum, a weak positive serum, and a negative serum. The highest dilution giving a 4+ reaction was 1:24 with the strong positive serum and 1:9 with the weak positive serum, while there was no reaction in any of the dilutions with the negative serum. A 1:6 dilution of the stock antigen was selected for routine testing of samples. Therefore, the final concentration of antigen was 0.555 mg./cc. of suspension.

The antigen prepared at Louisiana in 1952 was titrated in the same manner as the Maryland antigen. The primary antigen suspension contained 5 mg./cc. Since a titer of 1:24 was obtained with strong positive serums and no titer with negative serums, a dilution of 1:12 was selected as optimal. The final concentration employed in the test was 0.416 mg./cc.

The actual testing was conducted by setting up two tubes for each sample, as well as for the positive and negative control serums. Three-tenths cubic centimeter of veronal-bicarbonate buffered salt solution was pipetted into one of each pair of tubes, then 0.3 cc. of a 1:10 dilution of serum was added to both tubes. To the tube containing no diluent, 0.3 cc. of the antigen suspension was added. Three-tenths cubic centimeter of the optimal dilution of complement (determined previously that day) was pipetted into all tubes. An antigen control tube containing 0.3 cc. of veronal-bicarbonate buffered salt solution, antigen, and complement was also run with the test. The tubes were shaken and incubated for one hour at 37 C. in the water bath.

An equal volume of the proper amboceptor dilution was mixed with the 2 per cent cell suspension. Six-tenths cubic centimeter of this mixture was added to all tubes. The tubes were again shaken and reincubated for thirty minutes at 37 C.

Visual determination of results was carried out by estimating the degree of fixation. A tube containing a fairly heavy suspension of cells, which permitted little or no passage of light, was recorded as a 4+ reaction, while a tube giving a slightly turbid appearance with a very small button of cells on the bottom was recorded as a 1+. Reaction intermediate to these extremes were recorded as either 2 + or 3+.

In order to make spectrophotometric determinations, it was necessary to add sufficient diluent to the tubes to increase the volume to the point where it could be satisfactorily read in the instrument being used (0.5 cc. in the case of the 12 x 75 mm. cuvettes of the Coleman Jr. spectrophotometer). After the diluent was added, the tubes were centrifuged for five minutes at 2,000 r.p.m. and read using the optical density of a completely hemolyzed sample as the 100 per cent hemolysis control. As there was variation in readings of samples that appeared to be completely hemolyzed, it was decided that all samples showing 91 to 100 per cent hemolysis be considered as showing no reaction.

The 77 samples from Maryland herds were sent to Miller at Louisiana and Brock at Oklahoma; the 92 samples from Louisiana herds were sent to Price at Maryland and Brock; and the 109 Oklahoma samples were tested by the Maryland and Louisiana investigators. Histories were not sup-

plied to the investigators until all testing was completed.

Since, up to this time, no clear cut data were available concerning interpretation of test readings, each investigator used his own standards for the interpretation of results. He also had the option of deciding which set of results (visual or spectrophotometric) should be emphasized in arriving at an interpretation.

RESULTS

Table 1, summarizing the results of both the visual and spectrophotometric readings, presents the results of 834 visual and 834 spectrophotometric readings since the 278 serum samples were tested by each of the three investigators. The results obtained by testing serums from animals with positive histories are separated from those obtained with serums from negative animals. The figures in the table indicate the number of samples which gave a particular visual or spectrophotometric reading. For example, in the visual reading of the positive samples, 192 of the 225 positive samples that were given a correct interpretation produced 4+ reactions, while 23 gave a 3+ reaction. Three positive samples giving 4+ reactions were wrongly interpreted as negative, as were 2 samples that gave 3+ reactions. The final interpretation of results was based on readings by both visual and spectrophotometric methods. In some cases, greater emphasis was placed on spectrophotometric readings, hence these 3+ and 4+ visual readings that were given a negative interpretation. Only 4 of the 21 positive samples which were given wrong interpretation produced less than a 1+ reaction.

Of the 577 negative samples that were given the correct interpretation, 527 gave no reaction and 32 gave a 1+ reaction. A total of 18 samples gave readings ranging from 2+ to 4+. Ten of the eleven negative samples that were incorrectly interpreted gave readings greater than 1+.

As the spectrophotometric results indicate, 220 of the 225 correctly interpreted positive samples produced 0 to 70 per cent hemolysis, while all 21 of the incorrectly interpreted samples showed greater than 70 per cent hemolysis.

Five of the negative samples (1 correctly interpreted, 4 incorrectly interpreted) produced less than 70 per cent hemolysis.

Twenty-one (8.5%) of the 246 positive samples were incorrectly designated as negatives, while 11 (1.8%) of the 588 negative samples were called positive. The total error was 3.8 per cent since 32 of 834 samples were given interpretations that did not coincide with the histories of the animals from which the samples were obtained.

TABLE 1—Compilation of Visual and Spectrophotometric Readings

	Positive samples		Negative samples	
	Correct interpretation	Wrong interpretation	Correct interpretation	Wrong interpretation
VISUAL READINGS				
.....	0	4	527	0
1+	0	7	32	1
2+	10	5	11	7
3+	23	2	4	2
4+	192	3	3	1
Total	225	21	577	11
SPECTRO. READINGS (%)				
100-91	0	11	543	0
90-81	1	5	26	0
80-71	4	5	7	7
70-61	8	0	1	0
60-51	9	0	0	3
50-41	16	0	0	0
40-31	13	0	0	0
30-21	29	0	0	1
20-11	58	0	0	0
10-0	107	0	0	0
Total	225	21	577	11

Brock, placing greater emphasis on visual readings and using an antigen concentration of 0.555 mg./cc., gave the proper interpretation to all positive samples. He recorded a total of 7 false positives.

An antigen concentration of 0.416 mg./cc. was employed by Price. He also placed more emphasis on visual readings in arriving at an interpretation. Four false positives and 5 false negatives were obtained.

The antigen suspension used by Miller contained 0.416 mg./cc. He emphasized spectrophotometric readings. His results showed 16 false negatives.

DISCUSSION

The results in table 1 indicate that despite the lack of uniformity employed in the interpretation of test readings, an accurate differentiation could be made between carrier and noncarrier animals. Although the total percentage of error (3.88) was well within the range found in other widely accepted serological tests, the

greater part of this error occurred with positive serums. The total percentage of error in the testing of positive samples (8.5) can not be regarded as the actual

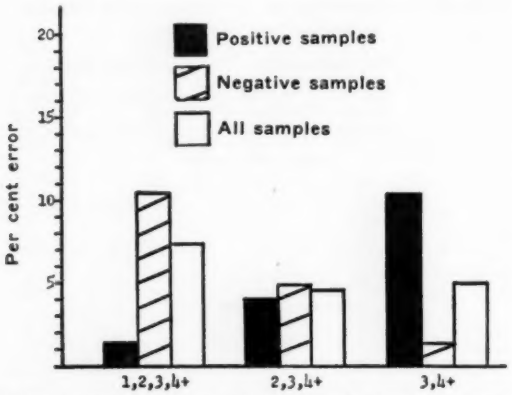


Fig. 1—Comparison of standards for interpretation of visual readings.

error involved in the test because of the discrepancies produced by the different methods of interpretation.

The data shown in figures 1 and 2 were calculated in an effort to establish a definite line of demarcation for interpretation of visual and spectrophotometric readings. Each investigator was permitted to make his interpretations according to his own particular standards since no other data were available as to which readings should be given a positive and which a negative interpretation. Figure 1 contains the data calculated from visual readings, while figure 2 gives the data based on readings obtained by spectrophotometric methods. The figures show the percentage of error that would have been obtained if the line of demarcation for interpretation of results had been drawn at a particular degree of fixation. For example in figure 1, if all animals whose serums gave 1+, 2+, 3+, and 4+ reactions had been called reactors, then 4 (1.6%) of the 246 positive samples would have been given the wrong interpretation, while 61 (10.3%) of the 588 negative samples would have been called positive. The total error would then have been 7.7 per cent.

That same pattern can be observed in figure 2. If a standard is selected that decreases the percentage of error of the positive samples, an increase will be created in the percentage of error of the negative samples. There was only one standard (0.90% hemolysis given a positive interpretation) that would have reduced the percentage of error with positive samples to less than 5.0. This same standard would have given 7.5 per cent error with the negative samples and an overall percentage of error of 6.6.

Spectrophotometric readings for the most part gave just as accurate an index of the carrier status of the animals as did the visual results. However, there were some instances where visual readings of 1+, 2+, 3+, and even 4+ gave greater than 90 per cent hemolysis when read spectrophotometrically.

The great discrepancies found in some cases between the visual and spectrophotometric readings for the same sample were probably due to a combination of time and dilution. Visual readings were made immediately following removal of the tubes from the water bath, while in some cases, due to the large number of samples involved and to the manipulations required to get the sample in suitable condition to make the reading, thirty to forty-five minutes elapsed before the last of the spectrophotometric readings was completed. With some samples, the hemolytic activity of the complement apparently continued to function during this interval. This factor could probably be kept at a minimum if the samples were placed in the water bath in a staggered fashion so that only a small number of samples would have to be read at one time. Chilling of the samples following removal from the water bath or removal of

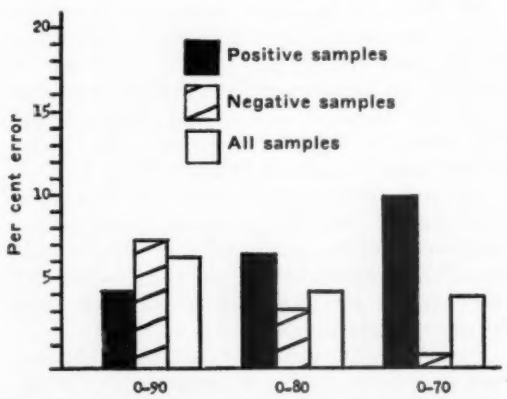


Fig. 2—Comparison of standards for interpretation of spectrophotometric readings.

the supernatant liquid following centrifugation would also serve to prevent further hemolysis.

Since the instruments employed for the spectrophotometric determinations required a minimum volume of 2.0 cc. to obtain satisfactory readings and since the total volume used in testing was only 1.5 cc., it became necessary to add sufficient diluent to make up this deficit. At Louisiana State University, Miller has shown that the addition of diluent will in some cases cause additional hemolysis, whereas no increase could be detected when the volume of test ingredients was increased to the point where no diluent was necessary. The titer of the serums may have been responsible for the fact that good correlation was obtained with some samples and not with others. High titer serums in combination with antigen probably form a more stable complex with the complement, thus reducing the effect of the added diluent to a minimum, whereas low titer serums and antigen form a less stable complex with complement. The added diluent may be instrumental in releasing complement from this complex, thus causing additional hemolysis. If spectrophotometric determinations were to be employed on a routine basis, it would be necessary to increase the aliquots of test ingredients so that the total volume would be 2.0 cc. or use an instrument requiring not more than 1.5 cc.

The most satisfactory standards of interpretation of results would appear to be as follows: visual readings of 2+, 3+, and 4+ to be considered positive; 1+ readings to be called suspicious and retested. If a 1+ reaction is again obtained, a new serum sample should be drawn and tested. Spectrophotometric readings of 0 to 80 per cent hemolysis to be considered positive; 81 to 90 per cent hemolysis to be called suspicious and retested as suggested for 1+ visual readings; 91 to 100 per cent hemolysis to be considered negative.

The concentration of antigen employed by the various investigators showed a close relationship to the number of reactors missed and to the number of false positives obtained by each of them, as mentioned in the section on results. This difference can readily be explained on the basis of the different standards used by the investigators in arriving at an interpretation. Miller, who emphasized spectrophotometric readings in his determinations, would have had only 6 missed reactors if he had emphasized visual readings as did Price and Brock; that is, if he had considered all samples giving 2+, 3+, or 4+ readings as positive.

It would appear from these results that the absolute strength of the antigens prepared at the three institutions was approximately the same. The difference in the antigen concentration that was selected by each investigator as being optimum can be explained only by the antigen titration method employed.

Since it has long been established that a deficiency of either antigen or antibody can be partially overcome by an excess of the other component, this undoubtedly is the explanation for the larger number of carriers missed by Miller and Price and points up the merit of the antigen titration method utilized by Brock. For this reason, we feel that this antigen titration procedure should be adopted for all anaplasmosis complement-fixation tests.

Summary

Data from complement-fixation testing by three laboratories of 824 samples of bovine serums of known anaplasmosis history show that the accuracy of the test is 96 per cent in identifying anaplasmosis carrier cattle.

Visual and spectrophotometric methods of reading the test results were used. Under conditions of this experiment, visual readings were more accurate. Improvements in technique are suggested which may increase the accuracy of spectrophotometric readings.

Suggested standards for the interpretation of test results are as follows: visual, 2+, 3+, and 4+ positive, 1+ suspicious; spectrophotometric, 0 to 80 per cent hemolysis positive, 81 to 90 per cent hemolysis suspicious, 91 to 100 per cent hemolysis negative. Suspicious samples should be retested or another sample should be taken and tested if necessary.

The antigens used in this experiment were prepared at each of the three laboratories by the method of Price, Poelma, and Faber [5] and were found to be of about the same relative strength. The optimal concentration of these antigens lay near 0.555 mg./cc. of antigen suspension.

References

[1] Rees, C. W., and Mohler, W. M.: Preliminary Studies on the Complement-Fixation Test for the Diagnosis of Bovine Anaplasmosis. J.A.V.M.A., *85*, (1934): 669-674.

[2] Boynton, W. H., and Woods, G. M.: A Serum Reaction Observed in Anaplasmosis. J.A.V.M.A., *87*, (1935): 59-63.

[3] Mohler, W. M., Eichhorn, E. A., and Rogers, H.: Complement-Fixation Test for Serum Diagnosis of Bovine Anaplasmosis. Vet. Med., *44*, (1949): 155-156.

[4] Mott, L. O., and Gates, D. W.: The Production of an Antigen for Anaplasmosis Complement-Fixation Tests. Vet. Med., *44*, (1949): 296-299.

[5] Price, K. E., Poelma, L. J., and Faber, J. E.: Preparation of an Improved Antigen for Anaplasmosis Complement-Fixation Tests. Am. J. Vet. Res., *13*, (1952): 149-151.

[6] Price, K. E., Poelma, L. J., Hastings, J. W., Sr., and Mitchell, J. I.: The Practical Application of the Complement-Fixation Test for Anaplasmosis. Proc. U. S. Livestock San. A., (1953): 90-97.

[7] Kent, J. F., Bukantz, S. C., and Rein, C. R.: Studies in Complement-Fixation. I. Spectrophotometric Titration of Complement; Construction of Graphs for Direct Determination of the 50 per cent Hemolytic Unit. J. Immunol., *53*, (1946): 37-50.

[8] Kent, J. F.: An Abbreviated Technique for Determining the Optimal Concentration of Amboceptor. J. Lab. Clin. Med., *31*, (1946): 1270-1277.

Statement of Ownership and Management Required by the Acts of Congress of August 24, 1912, as amended by the Acts of March 3, 1933, and July 2, 1946, for the American Journal of Veterinary Research. Published Quarterly at Chicago, Ill., as of Oct. 1, 1948: "State of Illinois, County of Cook, before me, a notary public, in and for the state and county aforesaid, personally appeared Dr. J. G. Hardenbergh, who having been duly sworn according to law, deposes and says that he is the managing editor of the American Journal of Veterinary Research and that the following is to the best of his knowledge and belief a true statement of the ownership and management of the aforesaid publication for the date shown in the above caption, required by the Act of August 24, 1912, as amended by the Act of March 3, 1933, embodied in section 537, Postal Laws and Regulations, to wit:

"(1) That the names and addresses of the publisher, editor, managing editor, and business manager are:
Publisher—American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.
Editor—W. A. Aitken, 600 S. Michigan Ave., Chicago 5, Ill.
Managing Editor—J. G. Hardenbergh, Chicago 5, Ill.
Business Manager—J. G. Hardenbergh.

"(2) That the owner is: American Veterinary Medical Association (a non-profit corporation), 600 S. Michigan Ave., Chicago 5, Ill.; Abner H. Quin, President, P. O. Box 167, Kansas City, Mo.; Harry E. Kingman, Jr., Treasurer, 600 S. Michigan Ave., Chicago 5, Ill.; J. G. Hardenbergh, Executive Secretary, 600 S. Michigan Ave., Chicago 5, Ill.

"(3) That the known bondholders, mortgagees, and other security holders holding 1 per cent or more of total amount of bonds, mortgages, or other securities are: None.

"(4) Paragraphs 2 and 3 include, in cases where the stockholder or security holder appears upon the books of the company as trustee or in any other fiduciary relation, the name of the person or corporation for whom such trustee is acting; also the statements in the two paragraphs show the affiant's full knowledge and belief as to the circumstances and conditions under which the stockholders and security holders who do not appear upon the books of the company as trustees, hold stock and securities in a capacity other than that of a bona fide owner.

(Signed) J. G. Hardenbergh."

"Sworn to and subscribed before me this Twenty-seventh day of September, 1954:
Helen S. Bayless, Notary Public (commission expires October, 1956)."

Antibodies for Hemolysins of *Micrococcus Pyogenes* in Whey and Serum of Dairy Cattle

JANIS LASMANIS, D.V.M., and G. R. SPENCER, D.V.M., Ph.D.

Pullman, Washington

A TRUE infection with tissue invasion by *Micrococcus pyogenes* in the mammary gland of dairy cattle should evoke an antibody response.

Varying titers of antihemolysins were reported in the milk and blood by Miller and Heishman,[1] but these titers were only roughly correlated with the presence of udder infection. Gwatkin[2] found 1.25 to 38.0 international units (I.U.) of staphylococcal antihemolysin antibodies per milliliter of serum of infected cows with the majority over 4 I.U. per milliliter. He also found small concentrations of antibodies, usually below 2.5 I.U per milliliter, in milk whey.

Minett[3] reported that blood contains 40 to 80 times as much antitoxin as milk whey. However, in colostrum and mammary exudates, the level of antitoxin might equal or exceed that in the blood of the same animal. Schalm and Woods[4] mentioned that rising titers in older animals may increase resistance to infection. They suggested this as an explanation for recovery or partial recovery of certain animals.

The present investigation was conducted to determine the relationship between milk whey or serum titers and the presence of infection.

MATERIALS AND METHODS

Freshly collected milk samples from individual quarters of 83 cattle in three herds were examined bacteriologically, employing procedures favorable for detection of *M. pyogenes*. Milk whey from the same samples was examined for antibody content. One year later, 64 cattle in two of these herds were retested. The same procedures were employed except that blood serum titers were also determined.

Bacteriological culture of milk entailed the direct streaking of a large loopful of freshly collected milk on the surface of Difco blood agar plates (8% bovine blood). These plates were incubated for forty-eight hours and examined. Only those quarters from which coagulose-positive organisms were isolated were regarded as infected.

Antihemolysin concentrations were determined by the method of Hartley and Smith.[5] A hemolytic toxin was obtained from the supernatant fluid of a 48-hour culture of a selected strain of *M. pyogenes*. This toxin was standardized with antitoxin supplied by the National Institutes of Health, Bethesda, Md.

The following method was employed to measure the concentration of antihemolysins. Milk samples were mixed with appropriate quantities of renin and incubated for one hour at 37 C. The whey was removed and inactivated at 57 C. for thirty minutes. It was then mixed with 0.85 per cent saline solution in successive twofold dilutions. Inactivated blood serums were diluted in the same manner as whey. Either 0.5 or 1.0 hemolytic unit of hemolysin contained in 0.5 ml. of physiological saline was mixed with 1 ml. of each dilution of whey or serum and incubated for one hour at 37 C. Then 0.5 ml. of a 4 per cent suspension of erythrocytes previously washed three times in saline solution was added to each tube. The mixture was incubated for one hour at 37 C. and refrigerated overnight at 5 C. The degree of hemolysis was observed and the end point or titer was regarded as the lowest dilution in which complete or almost complete hemolysis occurred. This titer was converted into international units (I.U.) per cubic centimeters of whey or serum by dividing the reciprocal of the titer by the units of toxin used in the test.

RESULTS

The antihemolytic titer of milk whey from infected and noninfected quarters is presented in table 1. Milk from noninfected cows usually showed little if any detectable antibody. Samples from noninfected quarters of infected cows had low titers in most instances, and their average titer was much lower than the correspond-

TABLE 1—Naturally Occurring Antihemolytic Titer Against Micrococcus Pyogenes in Whey Samples from Infected and Noninfected Cows

Concentration in I.U.*		<0.5	0.5	1	2	4	8 and more
Infected cows	No. infected quarters	11	24	42	33	13	3
	No. noninfected quarters	33	8	18	5	4	0
Noninfected cows	No. quarters	119	8	8	4	0	0
Total	No. quarters	163	40	68	42	17	3

* Titers expressed as international units of antitoxin per milliliter.

Scientific paper 1299, Washington Agricultural Experiment Station, Pullman, project 1023, supported in part by a grant from Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The authors are indebted to Dr. W. M. Dickson, Department of Veterinary Physiology and Pharmacology, for the statistical analysis, interpretation of data, and preparation of figure 1.

ing infected quarters of the same cows. The majority of infected quarters had 1 or 2 I.U. per cubic centimeters of whey. The maximum titer found in whey from an infected quarter was 32 I.U.

TABLE 2—Serum Titers of Antihemolytic Antibodies for Micrococcus Pyogenes in Infected and Noninfected Cows

	Titer in international units per milliliter					
	<0.5	0.5	1	2	4	8 and more
Infected	0	2	1	4	2	11
Noninfected	26	8	2	3	1	4
Total	26	10	3	7	3	15

The relationship of serum titer to the presence in the milk of coagulase-positive micrococci is indicated in table 2. The highest natural serum titer found was 64 I.U. per milliliter. Most infected cows had 2 or more I.U. of antihemolysin per milliliter of serum, whereas most noninfected cows had less than 2 I.U. A few noninfected cows had high titers of 8 I.U. or more. These may have had a previous infection or the methods used have failed to detect the infection.

The relationship between age and infection is indicated in table 3. It is based on data taken from two herds in which the average incidence of infection was 36.5 per cent. The incidence of micrococcus infection appears to have increased with age up to 5 years.

A correlation was found to exist between the pH of the milk and the staphylococcal antihemolysin titer of the whey. This is based upon studies of 92 milk samples from 23 cows. Examination of the data revealed that higher titers tended to occur in more alkaline samples. Statistical evaluation of this relationship in infected cows disclosed a very significant (P=0.01) positive linear regression of log titer (dependent variable) on pH (independent variable) (fig. 1). This regression was calculated using the mean of the four individual quarter samples for each cow. Moreover, if individual samples are used for calculation, a very significant positive regression with about the same slope was demonstrated.

TABLE 3—Cows Infected* with Micrococci by Age Groups

Age (years)	2	3	4	5	6	7 and over
Infected	0	1	3	11	4	8
Noninfected	5	14	12	3	6	7
Infected (%)	0	7	20	79	40	53

* Criterion of infection was the presence of coagulose-positive *Micrococcus pyogenes* in milk from one or more quarters.

DISCUSSION

The data presented above agree in general with those of Miller and Heishman [1] and indicate a definite correlation between the presence of infection and serum or whey titers of staphylococcal antihemolysins. The relationship is not an exact one, however, and numerous discrepancies were discovered between cultural results and antibody titers. It appears that the serum titer may be used as a diagnostic aid under conditions where repeated culture of milk samples is not feasible.

Serum titers were usually much higher

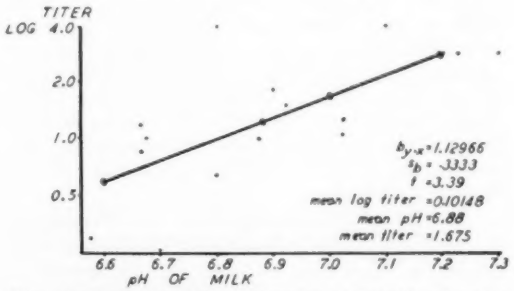


Fig. 1—Relationship between the pH of milk and the log of titer of staphylococcal antihemolysins.

than whey titers. Whey titers probably depend both on the serum titer and on the degree of permeability of the mammary tissues interposed between the blood and milk. Evidence for the importance of permeability is presented by the relation between the whey titers and the pH of the milk samples. In this interpretation, it is not the intention of the authors to exclude the possibility that some antibodies may be produced in mammary tissues. Since physiological factors, particularly the stage of gestation, affect the hydrogen ion concentration of milk, it is suggested that antihemolysin titers of whey be interpreted with caution. Serum antihemolysins appear to be more reliable as an indication of infection.

The relation of age to infection is manifest in a gradually increasing age incidence in these two herds. The data on serum and

whey titers do not support the concept that rising titers may be responsible for sufficient resistance to the disease to produce recovery from infection. On the contrary, high antihemolysin titers were usually accompanied by organisms shed into the milk. It is possible that antibodies other than antihemolysins may be concerned with resistance.

The degree of resistance associated with significant concentrations of antihemolytic antibodies may be a factor in preventing severe acute staphylococcal mastitis. The importance of the antihemolytic antibodies in chronic mastitis is doubtful and remains to be demonstrated. Sensitizing antibody in chronic staphylococcal mastitis inducing an increased susceptibility is also a possibility. A state of increased sensitivity in bovine streptococcal mastitis has previously been demonstrated by one of us.[6]

Summary

In a group of 126 quarter milk samples containing coagulase-positive staphylococci, only 11 had less than 0.5 international units of antitoxin and 49 had 2 or more units per milliliter of whey. In 207 quarter milk samples not containing coagulase-positive micrococci, 152 contained less than 0.5 units and only 13 had 2 or more units per milliliter. Similarly, serum titers in 34 of 44 noninfected cows were 0.5 units per milliliter or less, whereas serum titers were 2 or more units in 17 of 20 infected cows. These values suggest a trend toward lower titers in the animals culturally negative. There was a statistically significant correlation between the antibody titer of whey and the pH of the milk samples. This finding indicates that increased permeability of mammary tissues may be partly responsible for antibody in milk.

References

[1] Miller, W. T., and Heishman, J. O.: Staphylococcal Antitoxin in the Blood, Milk, and Colostrum of Cows. Am. J. Vet. Res., *4*, (1943): 265-269.

[2] Gwatkin, R.: Further Observations on Staphylococcic Infection of the Bovine Udder. Canad. J. Pub. Health, *28*, (1937): 185-191.

[3] Minett, F. C.: Staphylococcus Antitoxin in the Blood and Milk of Cows and Other Animals. J. Comp. Path. and Therap., *50*, (1937): 173-190.

[4] Schalm, O. W., and Woods, G. M.: *Micrococcus Pyogenes* in Bovine Milk. II. Relationship of Shedding Characteristics to Occurrence of Clinical Mastitis. Am. J. Vet. Res., *14*, (1953): 534-538.

[5] Hartly, P., and Lewellyn Smith, M. A.: A Proposed International Standard for Staphylococcus Antitoxin. Quart. Bull., Health Organization, League of Nations, Geneva, Special Number, (1935): 68-120.

[6] Spencer, G. R., and Angevine, D. Murray: Pathogenesis of Bovine Mastitis. III. The Significance of Hypersensitivity of Streptococcal Infection. Am. J. Vet. Res., *11*, (1950): 317-323.

An Outbreak of Histoplasmosis Involving Animals and Man

ROBERT W. MENGES, D.V.M.; MICHAEL L. FURCOLOW, M.D.;
ROBERT T. HABERMANN, D.V.M.

Atlanta, Georgia, and Bethesda, Maryland

A SUMMARY of the 13 outbreaks of histoplasmosis which have occurred in human beings has been presented by Furcolow *et al.*[1] and Grayston *et al.*[2] However, no data were presented concerning infection in the domestic animals in the vicinity. A recent outbreak[3,4] which occurred on a farm near Atchison, Kan., has afforded an opportunity for such a study.

The family involved included the mother, father, two sons, and the father's brother. Their ages in years were 30, 27, 6, 3, and 16. On Aug. 15, 1952, after living at their grandfather's farm three miles away for a year, the family moved to a farm which had been idle for two years, taking 2 collie dogs (5-year-old male and 4-year-old female) and 3, 1-year-old cats which they had raised.

On August 22, the 16-year-old boy cleaned the chicken house, and the manure was spread on the fields. The family helped, all being exposed to the dust. All developed an influenza-like illness by August 30, having fever, headache, and anorexia. The 16-year-old boy had a severe respiratory illness which required seventy-two days of hospitalization. Radiographs of each person showed lung lesions, the most severely ill showing disseminated lesions. All had positive histoplasmin skin tests and positive serological tests for histoplasmosis. *Histoplasma capsulatum* was not isolated from any of these persons.

Later in August, 2 young cows, a 6-month-old calf, and 27, 5-month-old pullets, were brought from the grandfather's farm.

On October 20, the bitch had 8 pups, and in November a Cocker Spaniel pup was acquired, bringing the number of animals on the farm to 44.

On November 13 and December 9, soil samples were obtained from inside and outside the chicken house (fig. 1 and 2). *Histoplasma capsulatum* was isolated from two samples from inside and from one outside near the door.[5,6] *Histoplasma capsulatum* was also isolated April 14, 1953, from air samplings taken 2 ft. off the ground floor inside the chicken house and 6 in. off the ground on the north side of the chicken house 6 in. from the building.

The purpose of this paper is to present the results of studies on 12 animals associated with this outbreak. In addition, the histoplasmin sensitivity rates among animals within a 2-mile radius of this farm are presented.

From the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta, Ga.; and the Pathology Laboratory, National Institute of Arthritis and Metabolic Disease, National Institutes of Health, Bethesda, Md.

MATERIALS AND METHODS

Of the 12 animals studied, all except 1 chicken were obtained alive and all were from this farm except 1 chicken from the grandfather's farm. The 5 pups and 1 cat were obtained for study Jan. 27, 1953, the 3 kittens on April 21, 2 chickens on Dec. 9 and 11, 1952, and the chicken from the grandfather's farm on June 11, 1953.

The chickens and kittens were soon destroyed, but 4 of the pups and 1 cat were first observed for three weeks. The fifth pup was given to a laboratory worker and observed for five months.

The animals on this farm and on nine nearby farms were skin-tested using a method that has been described.[7] Histoplasmin lot H (KC) 3 and 4, undiluted and diluted 1:10, lot H (KC) 7, diluted 1:10, and lot H (KC) D-2770, undiluted, were used as antigens.

Serums from several animals were tested for histoplasmosis using three tests:* (*a*) a complement-fixation test with histoplasmin[8] and yeast-phase[9] antigens; (*b*) a precipitin test with histoplasmin[10]; and (*c*) serums from 1 chicken and 3 cats were tested using an indirect complement-fixation test with histoplasmin.[11,12]

Histopathological and bacteriological studies were done on tissues from the 12 animals. Portions of lung, liver, spleen, adrenal, mandibular lymph node, proventriculus, gizzard, kidney, bladder, ovary, small intestine, and heart were ground and cultured on one brain-heart infusion agar (with 10% blood) plate and on two Sabouraud's glucose agar plates. The medium contained 40 units of streptomycin and 20 units of penicillin per milliliter. The plates were incubated at 25 C., and observed for a minimum of one month.

Portions of lung lesions and abdominal fluid of the dogs, and of gizzard contents, large intestine, feces, liver, spleen, lung, and small intestine of the chickens were inoculated intraperitoneally into mice. The mice were necropsied one month later, and the combined liver, spleen, and adrenal tissues were cultured using the same method employed for the original tissues.

* Appreciation is expressed to Dr. S. B. Salvin, Rocky Mountain Laboratory, Hamilton, Mont., and Dr. J. H. Schubert, Communicable Disease Center, Chamblee, Ga., for doing the serological tests.

RESULTS

Histoplasmin Tests.—On Dec. 9, 1952, the 44 animals on the farm were skin-tested with histoplasmin, 2 cattle and 1 chicken (7 %) reacting. One of the 3 cats tested could not be found when the tests were read but was later found dead.

TABLE 1—Histoplasmin Sensitivity Rates Among Animals on Farms Within a Two-Mile Radius of the Affected Farm at Atchison, Kansas

Animal	No. farms	Age (years)											
		Under 2			2 to 7			8 and over			Total		
		T	+	%	T	+	%	T	+	%	T	+	%
Cattle	10	16	0	0	90	5	6	7	1	14	113	6	5
Mules	1	0	0	0	0	0	0	2	1	50	2	1	50
Chickens	2	28	2	7	0	0	0	0	0	0	28	2	7
Dogs	2	9	0	0	2	0	0	1	0	0	12	0	0
Cats	2	5	0	0	0	0	0	1	0	0	6	0	0
Total		58	2	3	92	5	5	11	2	18	161	9	6

T—total tested; +—positive; %—% positive.

Since the 5 Collie pups and the cat studied had spent quite a bit of their time playing around the chicken house, they were skin-tested several times using various lots of histoplasmin, undiluted and diluted 1:10. Four of the pups developed positive skin tests when undiluted histoplasmin was used but failed to react when histoplasmin was diluted 1:10. The skin tests on the other pup and the cat remained negative.

On June 9, 17 cattle and 1 chicken were skin-tested on the grandfather's farm. The cattle were all negative, but since the chicken was positive it was taken for study. On June 11, when skin-tested again, it was positive at twenty-four hours.

During July and August, 1953, 161 animals on farms within a 2-mile radius of the farm were skin-tested and 9 (6 %) were found positive (table 1).

Serological Tests.—Serums from the 5 pups, 1 cat, 3 kittens, and 2 chickens, as well as from the 3 cattle on the farm, were tested using the complement-fixation test with histoplasmin and yeast-phase antigens and also with the precipitin test with histoplasmin. The 3 kittens and 3 cattle were negative.

The cat was positive 1:16 in the indirect complement-fixation test. One chicken was positive 1:8 in the indirect complement-fixation test, and positive 1:5 in the precipitin test. Four of the pups had positive and 1 a doubtful complement-fixation test using yeast-phase antigen, but all 5 had negative precipitin tests. When the pups were retested, 3 had positive and 2 doubtful complement-fixation tests, only 1 showing a rise in titer; 2 had positive precipitin tests.

Clinical Observations and Gross Pathology.—After the 5 pups and the cat had been observed for three weeks, all but 1 pup were destroyed for necropsy. The pups appeared to be normal although all were passing adult tapeworms and ascarids for which they were treated January 30. The cat had a severe diarrhea and loss of appetite for a few days before being subjected to necropsy. The remaining pup appeared normal until May 12, when it showed a loss of appetite. On May 14, its sclera were congested; its rectal temperature 101.4 F., after which it appeared to be normal until found dead in its kennel on June 8, with evidence of a hemorrhage at the nose prior to death.

The 3, 3-week-old kittens obtained from the farm on April 21 had been moved by the dam, when 1 week old, into a manure spreader and later to a feed box in the cow barn. They appeared normal but were obtained for study primarily to see whether animals could be infected shortly after birth; however, no evidence of infection was found.

Of the 12 animals destroyed for necropsy, none showed extensive gross lesions;

Fig. 1—The chicken house on the farm near Atchison, Kan. Histoplasma capsulatum was isolated from the soil near the door outside the chicken house.

the cat and 3 kittens had no gross lesions. Three pups had clear fluid in the abdominal cavity, the amount ranging from 100 to 500 cc., and 4 pups had numerous pin-point white lesions throughout the lungs and a

Fig. 2—Inside the chicken house. Histoplasma capsulatum was isolated from two soil samples from inside the chicken house.

few nodular lesions approximately 2 mm. in size on the surface of the lungs. The nodular lesions appeared to have a clear central portion. Two pups had kidney lesions, 1 a small white area beneath the surface of the kidney, the other a white lesion, 1 mm. in size, on the surface of the kidney.

Upon necropsy June 8, the fifth pup revealed the following lesion: liver enlarged with mottled areas, spleen approximately twice normal size, kidneys edematous, mesenteric and mandibular lymph nodes enlarged, and lungs hemorrhagic with multiple nodular lesions 3 to 5 mm. in size (fig. 5).

No symptoms had been observed in the chickens. While one of the 2 chickens from the farm showed no gross lesions, the other, the histoplasmin reactor (fig. 3), had an enlarged liver with one 2-mm. nodular lesion with a caseated center raised above the surface and one yellowish area at the edge. The spleen, somewhat enlarged, showed greyish mottled spots. The ovary was congested. Death was due to hemorrhage, the pericardial sac being filled with blood. The chicken from the grandfather's farm had one lesion, a dark 8-mm. area with a light center raised above the surface of the liver (fig. 6).

Histoplasma capsulatum was isolated from the tissues of 6 of the 12 animals. The fungus was isolated from the liver, spleen, and kidney of 1 pup; liver, lung, and mandibular lymph node of another pup; and from the liver, spleen, and lung of the remaining 3 pups and the cat.

Histopathological Findings.—The lung tissues from the 5 pups showed 1 with pneumonia and 4 with scattered small discrete nodular lesions measuring from 100 to 500 μ in diameter. One pup showed peribronchial and intraalveolar lymphocytic nodules, slight thickening of the alveolar septum, some infiltration of lymphocytes, and a few polymorphonuclear leukocytes. Three pups showed peribronchial and alveolar nodules with necrotic centers surrounded by dense strands of fibrous connective tissues infiltrated with lymphocytes. Some of the larger nodules also showed a central deposition of calcium. The livers from 2 pups had lesions, 1 showing scattered areas of focal necrosis and small nodular lesions infiltrated with lymphocytes and monocytes, the other showing several small granulomatous lesions made up of large mononuclear cells, lymphocytes, and fibroblasts. Two pups showed areas of focal necrosis in the tracheal lymph nodes, 1 showed areas of focal necrosis in one cer-

Fig. 3—White Plymouth Rock pullet, 9 months old, a reactor to 0.1 ml. of histoplasmin, lot H(KC) 3 and 4, undiluted. Reaction, 8 mm.; site of injection, right wattle.

vical lymph node, and 1 showed slight scarring of several of the lymph follicles in the colon.

The cat tissues showed peribronchial and perivascular lymphocytic nodules, several

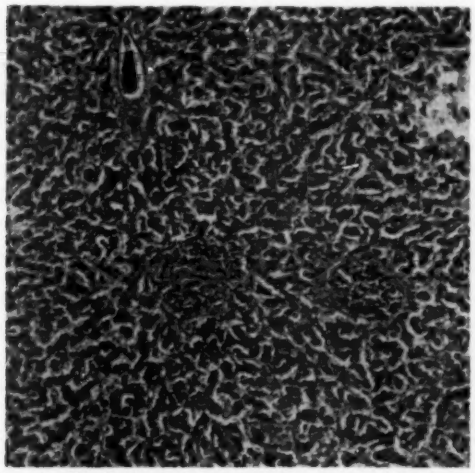


Fig. 4—Nodular liver lesions from the same chicken. Hematoxylin and eosin. x 87.

smaller ones, measuring 200 μ in diameter, compressing the alveoli; thin ribbon-like streaks of lymphocytes between the myocardial fibers; and small irregular areas of calcium deposition in the cells of the adrenal cortex. These areas were separated into small lobules by dense strands of fibrous connective tissue. The capsule of the adrenal gland showed fibrosis and the attached fascia was thickened by dense and wavy fibrous connective tissue.

Tissues of 2 of the chickens showed scattered small nodules made up of lymphocytes and macrophages and slight degeneration of myocardium in 1 of the birds. The liver of 1 showed perivascular infiltration of lymphocytes and one area showed two lymphocytic nodules surrounded by strands of fibrous connective tissue infiltrated with lymphocytes and inflammatory cells (fig. 4). The omental attachments of the small intestine of 1 chicken showed several fibrous areas infiltrated with inflammatory cells and macrophages, and the other chicken showed a fibrinous exudate, infiltrated lymphocytes, and macrophages containing yellowish brown pigment on the serosal surface of the small intestine. The omentum of both these chickens showed scattered irregular areas of fibrosis and infiltration of inflammatory cells and macrophages containing yellowish brown pigment. One kidney showed a lesion 200 μ in diameter made up of inflammatory cells. Although *H. capsulatum* was not isolated from the chickens, the histopathological lesions in the liver and heart were similar to those observed in chickens experimentally inoculated with spores of *H. capsulatum*.[13]

Discussion

This outbreak of human histoplasmosis appears to be the first reported in which animals in the vicinity of the point source have been found infected. There appeared to be no evidence that the animals were transmitting the infection. Most of the animals were either brought to the farm or born there after the family became sick.

Since *H. capsulatum* was isolated from the soil and air in the chicken house, it appeared that all the infection had come from this common source. Recent demonstration of *H. capsulatum* spores in the open air 75 ft. above the ground by Rooks[14] presents further evidence that infection occurs through the inhalation of air-borne spores.

The study of 5 of the pups which were born on the farm and infected early in life, presented several interesting facts. Two had positive histoplasmin skin tests at

Fig. 5—Multiple nodular lesions in the lung of the dog that expired at 7½ months of age. x 2.25.

3 months of age, 2 more were positive at 4 months of age, and 1 remained negative. The one that remained negative expired at 7½ months of age, revealing many lesions. The pups were skin-tested several times, but no positive reactions were obtained when histoplasmin diluted 1:10 was used. This can be explained by presenting the results of skin tests on 5 rabbits that were inoculated by an air-borne method with spores of *H. capsulatum*.[15] These 5 rabbits were followed until the 150th postinocula-

Fig. 6—Lesion in the liver of the chicken from the grandfather's farm. x 2.

tion day, all developing a positive skin reaction. On the thirty-second day, 2 reacted to histoplasmin diluted 1:10 but on the 38th, 52nd, and 73rd days, all failed to react to histoplasmin diluted 1:10, and only 1 reacted to undiluted antigen. Later when tested with histoplasmin diluted 1:10, 3 were positive on the 110th day, 4 on the 124th day, and all 5 on the 150th day. During the first one hundred days, intermittent fever occurred indicating a systemic reaction which probably suppressed the skin reaction. Since the pups were tested during the early phases of the infection (50 to 120 days after birth) the skin reaction may likewise have been suppressed by a systemic reaction or possibly by other factors.

The histopathological studies revealed calcified nodules in the lungs of the pups and deposition of calcium in the adrenal gland of 1 cat.

SUMMARY

A description of an outbreak of histoplasmosis among human beings and animals is presented. A total of 12 animals were studied including 5 dogs, 4 cats, and 3 chickens. Their clinical symptoms, necropsy, and cultural findings, histoplasmin skin test results, and serological test results are presented. In the 12 animals studied, *Histoplasma capsulatum* was isolated from 5 dogs and 1 cat. The fungus was not isolated from the 3 chickens but 2 were histoplasmin reactors. A total of 161 animals within a 2-mile radius of the involved farm were skin-tested with histoplasmin and 9 (6 %) were found positive.

References

[1] Furcolow, M. L., and Grayston, J. T.: Nontuberculous Chest Diseases. Occurence of Histoplasmosis in Epidemics. Tr. Nat. Tuberculosis A., (1952): 83-91.

[2] Grayston, J. T., and Furcolow, M. L.: The Occurrence of Histoplasmosis in Epidemics. Epidemiological Studies. Am. J. Pub. Health, *43*, (1953): 665-676.

[3] Furcolow, M. L., Menges, R. W., and Larsh, H. W.: Unpublished data, 1953.

[4] Menges, R. W., Furcolow, M. L., and Hinton, A.: The Role of Animals in the Epidemiology of Histoplasmosis. Am. J. Hygiene, *59*, (1954): 113-118.

[5] Ibach, M. J., Larsh, H. W., and Furcolow, M. L.: Isolation of *Histoplasma Capsulatum* from Air. Science, *119*, (1954): 71.

[6] Ibach, M. J., Larsh, H. W., and Furcolow, M. L.: Epidemic Histoplasmosis and Airborne *Histoplasma Capsulatum*. Proc. Soc. Exptl. Biol. and Med., *85*, (1954): 72-74.

[7] Menges, R. W.: The Histoplasmin Skin Test in Animals. J.A.V.M.A., *119*, (1951): 69-71.

[8] Tenenberg, D. J., and Howell, A.: A Complement-Fixation Test for Histoplasmosis. I. Technique and Preliminary Results on Animal Serums. Pub. Health Rep., *63*, (1948): 163-168.

[9] Salvin, S. B.: Complement-Fixation Studies in Experimental Histoplasmosis. Proc. Soc. Exptl. Biol. and Med., *66*, (1947): 342-345.

[10] Salvin, S. B., and Hottle, G. A.: Serological Studies on Antigens from *Histoplasma Capsulatum* Darling. J. Immunol., *60*, (1948): 57-66.

[11] Karrer, H., Meyer, K. F., and Eddie, B.: The Complement-Fixation Inhibition Test and Its Application to the Diagnosis of Ornithosis in Chickens and Ducks. I. Principles and Technique of the Test. J. Infect. Dis., *87*, (1950): 13-36.

[12] Gentry, R. F.: The Complement-Fixation Test. A Review of Its Application in Poultry Diseases. Proc. Book, AVMA, (1952): 281-285.

[13] Menges, R. W., and Habermann, R. T.: Experimental Avian Histoplasmosis. (To be published)

[14] Rooks, R.: Air-Borne *Histoplasma Capsulatum* Spores. Science, *119*, (1954): 385-386.

[15] Menges, R. W., Grayston, J. T., and Altman, P.: Unpublished data, 1953.

Virus Diarrhea in Cattle

JAMES A. BAKER, D.V.M., Ph.D.; CHARLES J. YORK, D.V.M., Ph.D.;
JAMES H. GILLESPIE, V.M.D.; GRAYSON B. MITCHELL, D.V.M.

Ithaca, New York

In 1946, a new febrile disease of cattle was reported by Olafson and co-workers.[1,2] They isolated the agent, reproduced the disease experimentally, and observed that this disease was a transmissible infection characterized by profuse diarrhea with certain clinical signs of illness, particularly ulcerations of the mouth, which resembled rinderpest. With the cooperation of Walker,[3] they proved conclusively by both cross-immunity and neutralization tests that there was no antigenic relationship between rinderpest and the new disease of cattle. Since Olafson and his co-workers were able to transmit this disease by infectious material which contained no visible organisms, they concluded that a virus was responsible and named the disease "virus diarrhea" (VD).

The two strains used in the present study were obtained in 1948 from sick cattle whose illness had been diagnosed as virus diarrhea and, on this basis, both agents were considered to be similar to the virus previously reported by Olafson as the cause of virus diarrhea. The original infectious material was not preserved and, therefore, was not available for exact comparison with the strains reported in this paper. The present paper is a report of studies with two strains of the VD virus, one isolated from a cow in New York State and the other from a cow in the State of Maine.

Strains Used for Study

In June, 1948, a young heifer from the vicinity of Ithaca, N. Y., was brought to the New York State Veterinary College for examination. Lesions suggestive of virus diarrhea were found at autopsy and the spleen from this animal was obtained through the courtesy of Dr. Peter Olafson. A portion of this spleen was weighed and made into a 10 per cent suspension in saline with a glass grinder. After coarse particles had settled, 1 ml. of the supernatant fluid was inoculated intravenously into another calf. This calf sickened, which indicated that an infectious agent had been transmitted. The calf was autopsied and portions of its spleen were placed in vials and kept under dry ice refrigeration. In further studies, this agent was called strain 1. Shortly afterwards, a specimen from the State of Maine was submitted with descriptive signs of illness which resembled those of virus diarrhea. From the various materials sent, brain was selected for the inoculum. As described above, a portion was made into a 10 per cent suspension and was inoculated into a calf. This animal sickened, was autopsied, and portions of the spleen saved. The infectious agent in this material from Maine was designated strain 2.

A comparison of strain 1 and strain 2 was made by cross-immunity tests. In each test, a calf was inoculated intravenously with 1 ml. of a 10 per cent suspension in saline of infected spleen and, for fourteen days thereafter, was observed for signs of illness. After this period of observation, the calf was reinoculated with the same strain as was done initially and again observed for fourteen days. It then was inoculated with the other strain. Each inoculum was tested for infectivity by inoculating another calf. These tests were repeated five times with each strain in complete reciprocal cross-immunity tests. The results are shown in table 1, where it can be seen that each strain immunized against itself and against the other.

Material that contained the infective agent was examined for the presence of organisms under the ordinary optical microscope using a variety of bacteriological stains, as well as Macchiavello's method and dark-field examination. No organisms were seen. Various mediums, such as blood agar slants, PPLO (Difco) medium containing various concentrations of serum, Schüffner's medium for Leptospira, and sodium thioglycollate medium were inoculated with infective material, placed at room temperature or at 37 C. and incubated for various periods of time. No growth of organisms occurred. In addition, the yolk sac of hen's eggs embryonated for five, six, or seven days were inoculated with 0.5 ml. of a 10 per cent suspension in saline of

TABLE 1—Comparison of New York Virus (Strain 1) and Maine Virus (Strain 2) by Cross-Immunity Tests in Calves

Immunizing Strain	Strain 1	Strain 2
1	0/5	0/5
2	0/5	0/5
None	10/10	10/10

Denominator indicates number of animals tested; numerator indicates number of animals showing signs of illness.

From the Veterinary Virus Research Institute, New York State Veterinary College at Cornell University, Ithaca.

Charles J. York is now director of Virus Research, Pitman-Moore Co., Indianapolis, Ind.

Grayson B. Mitchell, Captain in the Army of the United States, is on assignment to the Virus Research Institute.

spleen from an infected calf. After further incubation for five days, eggs were examined, films prepared from the yolk sacs and stained by Macchiavello's method. No forms resembling those of the psittacosis-lymphogranuloma group or rickettsias were seen.

Failure to demonstrate visible organisms led to the conclusion that a virus had been obtained but, since the infectious agent obtained by the original workers on the virus diarrhea was no longer available, no comparison was possible. However, since strains 1 and 2 were obtained from animals showing signs of illness similar to the published description of virus diarrhea and since they produced similar signs of illness in inoculated calves, these strains were considered as the causative agent of virus diarrhea.

PRODUCTION OF DISEASE

Method.—The cattle used for study varied in age from 1 week to more than a year, were of either sex, and of the usual dairy breeds. Some came from dairy herds in New York State and the others from an experimental herd maintained by the Virus Research Institute. This herd has been kept in strict isolation for many years to prevent accidental infection. Animals to be inoculated were placed in isolation units and allowed to adjust to their new environment for at least one week. During this period of observation, temperatures and leukocyte counts were taken and any abnormal animal was discarded, except 2 that had hyperkeratosis. In every instance, calves from the Institute herd adjusted without difficulty and their temperatures and leukocyte counts were in the normal range. Not infrequently the animals purchased elsewhere developed high temperatures or other signs of illness so that they could not be used.

After a period of adjustment in the isolation units, calves were inoculated by one of two methods: Each of 75 calves from dairy farms and 25 calves from the institute herd was inoculated intravenously with 1 ml. of defibrinated blood, 1 ml. of a 10 per cent suspension in saline of spleen from an infected calf or of serial dilutions thereof, or each of 5 calves from the institute herd was given an intranasal inoculation of 10 ml. of a 10 per cent suspension in saline of spleen from an infected calf. After inoculation, temperatures were taken twice daily and total leukocyte counts made once each day. Serums were collected before inoculation and after recovery. From some infected animals, blood was taken and defibrinated at the time of inoculation, and again two, four, eight, and ten days after inoculation, for measurements of virus concentration. In addition, calves were killed either two to three days, four to five days, or seven to eight days after inoculation. Their spleens were removed and tested for virus. Also from some calves, material from various organs was placed in 10 per cent formalin and saved for histological study. Results of infectivity tests are presented in table 2.

As can be seen in table 2, calves were infected by inoculations either intranasally or intravenously. Of the group of calves from dairy farms, 55 of 75 showed signs of illness (73 %) while all of the calves from the institute herd became ill (100 %).

TABLE 2—Results of Infectivity Tests in Calves Inoculated with Virus Diarrhea Virus

Source of calves	Route of inoculation	Response
Dairy farms	Intravenous	55/75
Institute herd	Intravenous	25/25
	Intranasal	5/5

Denominator indicates number of animals tested; numerator indicates number of animals showing signs of illness.

FEATURES OF THE DISEASE

Clinical Data.—From two to four days after calves were inoculated, a slight rise in temperature usually occurred as the first evidence of infection. Often this was detected at one temperature reading only. The following day a leukopenia sometimes was observed for about twenty-four hours, while the temperature receded for two to three days. During this initial response, the animals continued to eat and showed no visible signs of illness. This was followed by a second phase in which the rise in temperature was higher than in the first stage and lasted for two or three days. At the same time, a definite leukopenia developed in most of the calves, although in some cases an uninterrupted leukopenia was found from the time of the initial rise in temperature.

The amount of infectious material given and the route of inoculation seemed to bear a definite relationship to the incubation period. In general, calves which received a larger amount of inoculum showed signs of infection sooner while those inoculated intravenously also responded in a shorter time than the ones which were inoculated intranasally, although the second phase of illness did not seem to be affected by these factors. Age also influenced the thermal and leukocytic reactions. In general, calves less than 1 month of age showed little or no increased temperature in the first phase and only a slight increase in the second phase. Older calves up to 1 year of age showed the reaction described above. Cattle over 1 year of age showed a longer interval before onset of fever and a higher temperature in the first phase than in the second. Leukocyte counts were lower in the older animals than in the very young group.

In the second phase, animals quit eating and drinking and many showed marked depression. In some, increased lacrimation was observed and examination of the nostrils and oral mucosa showed a reddening, especially around the teeth. Ulcers in the mouth, reported to be common in the natural cases of this disease, were not seen generally in calves that were inoculated experimentally. Diarrhea, the most common feature observed in natural cases of the disease, developed in about 50 per cent of the calves purchased from dairy herds but only occasionally in the calves from the institute herd. None of the calves died except the 2 that had hyperkeratosis when placed on experiment. These animals died eleven and fourteen days after inoculation. The others began eating again after the end of fever. In figure 1 a typical clinical response is shown.

An interesting sequela was observed by Olafson in five herds that contained 95 animals. Abortion occurred in 20 of 35 bred heifers about one month after they had showed signs of infection with virus diarrhea. These animals were entirely negative when tests were made for brucellosis. In our work with 2 pregnant cows, 1 aborted about six weeks after infection while the other did not.

Concentration of Virus in Relation to Clinical Course of Illness.—The original workers on virus diarrhea reported a concentration of only 100 infectious doses per gram of spleen. In their study, material for test was taken during the height of fever, or seven to nine days after inoculation, in the second phase of the clinical course. After finding that the course of illness in calves was diphasic, presence and concentration of virus in blood and spleen of calves were measured at the time of inoculation and also at intervals of two, four, eight, and ten days after intravenous inoculation with 1 ml. of a 10 per cent saline suspension of spleen from an infected calf.

For measurements of virus in blood, samples were taken on the designated days from the jugular vein of a calf, defibrinated, distributed into several vials, and stored under dry ice refrigeration. In making a test, a vial of blood was thawed, tenfold dilutions were prepared, and 1 ml. of each dilution was given intravenously to a test calf. To measure the virus in the spleen, a calf was infected as usual and, at the time interval to be tested, was killed, portions of spleen removed and placed under dry ice refrigeration. In this test, the spleen material was thawed, weighed, and made into a 10 per cent suspension in saline with a glass grinder. The suspension was centrifuged at 1,500 r.p.m. for five minutes, the supernatant removed, and tenfold dilutions prepared in saline. Tests of each dilution

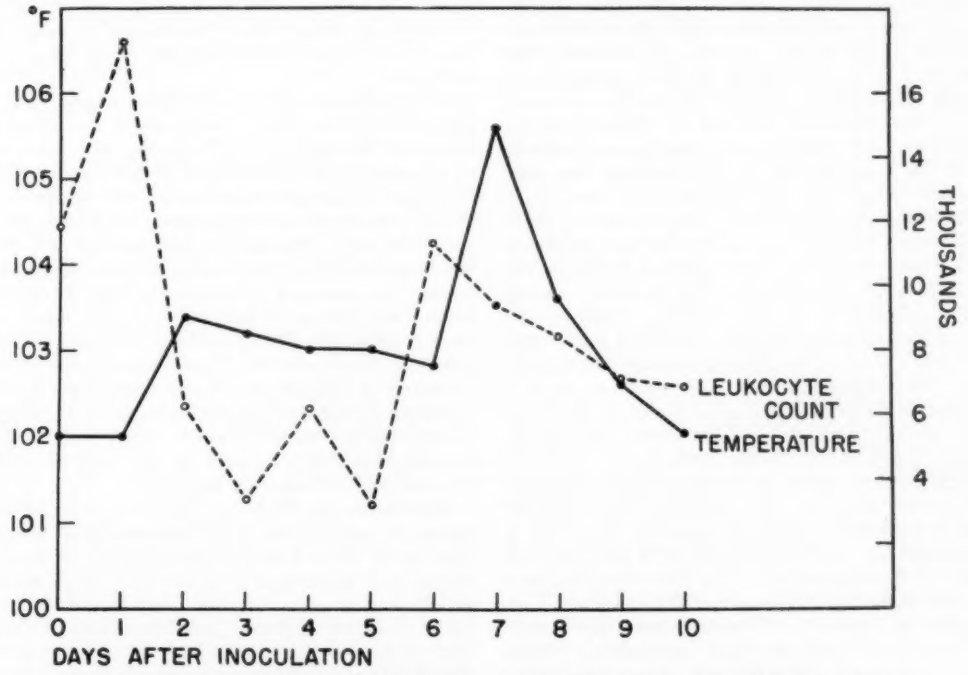


Fig. 1—Features of illness shown by calf inoculated with virus diarrhea virus.

were made in calves as for the blood dilutions. After inoculation, temperatures and leukocyte counts were made on the calves for fourteen days and then each calf was reinoculated with VD virus in a test for immunity. Thermal response, a leukopenia following initial inoculation, and no signs of illness when given the second inoculation constituted presence of the virus. The results are presented in table 3.

TABLE 3—Virus Concentration in Relation to Course of Illness

Days after inoculation	Infectious doses of virus	
	Blood	Spleen
0	None	None
2	10 to 100	100 to 1,000
4	1,000 to 10,000	10,000 to 100,000
8	10 to 100	100 to 1,000
10	None	Not tested

As can be seen in table 3, the greatest concentration of virus found in blood and spleen occurred four days after inoculation or in the interphase. Lesser concentrations were found two days and eight days after inoculation. No virus was found in the blood of 2 calves ten days after inoculation.

Pathological Conditions.—The material used for study was furnished by 21 calves, 2 of which died while the others were killed four to sixteen days after inoculation. From 5 of these animals, histological sections were prepared from liver, spleen, kidneys, lungs, and portions of the intestinal tract.

Autopsy examination showed no changes that could be attributed to virus diarhea except for 3 animals that showed ulcers in the mouth. Involvement of the esophagus reported by Olafson and coworkers was not seen in any of the calves. Reddening of the stomach and of the intestines was seen in some calves. The animals that died were the 2 which had been affected by hyperkeratosis before inoculation. In addition to the changes produced by this condition, they showed ulcers in the mouth and extensive pneumonia. Three other calves showed pneumonia in the anterior lobes of the lungs but the lesions appeared to be of long duration. Since these calves were purchased from dairy farms, the pneumonia may have occurred from a previous infection. Histological study of the liver, lungs, spleen, kidneys, and intestines showed no lesions. No inclusion bodies were seen.

Serological Studies.—In animal studies, the presence of VD virus in test material was indicated when inoculations produced signs of illness in a susceptible calf and failed to do so in one that had recovered from infection. Final confirmation was obtained when no illness occurred as the result of a second injection of VD virus into a calf which had recovered from the first inoculation. These tests for incidence of VD virus in cattle were exceedingly costly both in time and materials. A suitable tube test was sought and investigations were made of complement-fixation and neutralization tests.

Complement-Fixation Test.—In the preparation of antigen, spleen was removed from an animal killed four days after inoculation or at the height of febrile response and placed immediately under dry ice refrigeration. Portions of this spleen were thawed and homogenized in a Waring blendor to make a 20 per cent suspension by wet weight in buffered saline (pH 7.2). This preparation was then centrifuged at 1,500 r.p.m. to remove coarse particles, then formaldehyde was added to a final concentration of 0.2 per cent. Afterwards, the preparation was placed in a refrigerator at 4 C. for forty-eight hours and again centrifuged at 2,000 r.p.m. The supernatant fluid was saved and the sediment discarded.

Additional procedures in making antigen consisted of high speed centrifugation in order to concentrate antigenic activity. The material centrifuged consisted of a 20 per cent suspension of spleen in saline. The suspensions were divided into two portions: One portion was subjected to rapid freezing and thawing six times and the other left untreated. Both portions were then precipitated with protamine sulfate and centrifuged at 2,000 r.p.m. for ten minutes in a horizontal centrifuge. Some of the sediment was saved from each portion. The supernatant portion was centrifuged at 16,000 r.p.m. for one hour in a high speed angle centrifuge and the supernatant portion of this was carefully aspirated and discarded. The sediment was resuspended in a small amount of saline. All of the materials to be tested were lyophilized in small volumes and, when later reconstituted, served as antigens.

In attempts to standardize this antigen for complement-fixation tests, complement was first titrated in the presence of varying concentrations of antigen. Using 2 units of complement, twofold dilutions of antigen were tested for complement-fixing properties in the presence of bovine serum collected before inoculation and again two to four weeks afterwards. Each material was employed in 0.5-ml. amounts and thoroughly mixed. These mixtures were placed at 4 C. for eighteen hours and then a hemolytic system added that consisted of 2 per cent sheep cells sensitized with 2 units of ambocepter. Readings were made after a further incubation period of thirty minutes at 37 C. Appropriate controls were included. Tests that utilized serums from 2 to 5 animals for each antigen failed to show any evidence of fixation.

Neutralization Test.—In these tests, spleen shown to contain 1 ml. x 10^3 infective doses of virus was used. For virus preparation, a portion of spleen was made into a 10 per cent suspension in saline with a glass grinder, centrifuged at 1,500 r.p.m. for five minutes, the supernatant decanted and used to prepare dilutions. To 1.5 ml. of the 10^{-1} dilution an equal portion of unheated serum from an immune calf was added, thoroughly mixed,

and placed at 4 C. for two hours. Then, 2 ml. of the mixture was inoculated into a susceptible calf. In a similar manner, a 10^{-3} dilution was mixed with serum from a nonimmune calf, incubated, and inoculated into a calf. In another test, serum that had been heated at 56 C. for thirty minutes was used. All these tests were repeated. The calves became ill, which indicated the inability of either treated or untreated serums from immune calves to neutralize virus.

TRANSMISSION TO OTHER ANIMALS

In studies on infectious agents pathogenic for cattle, the work is greatly aided if smaller laboratory animals can be used for test purposes. Also, studies of this kind indicate host range, which is a consideration in epidemiological studies. In the search for a suitable species, all types of available laboratory animals were tested and, in addition, other farm animals and dogs were tested.

Eggs.—A 10 per cent suspension in saline of spleen from an infected calf was prepared and coarse particles allowed to settle. Into the yolk sac of each of a group of 5 hen's eggs, embryonated for five days, 0.5 ml. of the supernatant was inoculated. On the chorioallantoic membrane of each of another group of 5 eggs that had been embryonated for nine days, 0.1 ml. of the supernatant was inoculated. After an incubation period at 37 C. for five days, yolk sac membranes and chorioallantoic membranes from the inoculated eggs were removed, the membranes of each group pooled, made into a 10 per cent suspension and other eggs inoculated in the same manner as for the initial passage. After five serial transfers, 1 ml. of a 10 per cent yolk sac suspension was inoculated intravenously into a calf and 1 ml. of a 10 per cent chorioallantoic membrane suspension likewise inoculated into another calf. Neither calf became ill but when later given 1 ml. of spleen suspension from an infected calf, they did become ill, which indicated that virus had not been propagated either in the yolk sac or chorioallantoic membrane. This test was repeated twice and the conclusion was reached that this strain of VD virus would not infect eggs.

Guinea Pigs.—Each of a group of 3 guinea pigs, with an average weight of about 300 Gm., was inoculated intraperitoneally with 1 ml. of a 10 per cent suspension in saline of spleen from an infected calf. The guinea pigs were killed five days after inoculation, their spleen removed, pooled, made into a 10 per cent suspension and inoculated into a calf. Another group of guinea pigs was inoculated in a similar manner but each guinea pig was given 10 mg. of cortisone.[4,5,6] These animals also were killed five days after inoculation, a pool of their spleens made, suspended in saline, and inoculated into a test calf.

Neither calf became ill following the inoculation of guinea pig material. Each calf later was given a test inoculation and both showed signs of illness. It, therefore, appeared that VD virus could not be transferred to guinea pigs.

Rabbits.—The rabbits used were from mongrel stock, largely Polish-Dutch and were 3 to 5 months of age. For inoculation, each of 2 rabbits was inoculated intravenously with a 10 per cent suspension in saline of spleen from an infected calf. The rabbits were killed after five days and a portion of spleen from each was used for making another 10 per cent suspension. A calf was given 1 ml. of this suspension intravenously in a test for virus. This calf showed the usual signs of illness, was killed, a portion of its spleen removed, made into a 10 per cent suspension, and 2 rabbits were inoculated as for the first passage. Again, spleens were removed from these rabbits and inoculated into a calf to make the second alternation as was done when rinderpest virus was adapted to rabbits.[7] This calf also became ill, was killed, and its spleen was used to initiate serial passages in rabbits. Spleens from rabbits were tested for virus at the 5th, 10th, 15th, 30th, 50th, and 75th serial passage. Temperatures were taken twice daily on calves and once each day on rabbits. Total leukocyte counts were made daily on the calves. All animals that were autopsied were examined for lesions. Calves that received material from rabbits in the serial passages were reinoculated with virus that had been maintained only in calves. If the calf showed no signs of illness from this test injection, it was evident that VD virus had been present in the rabbit material used for the first inoculation.

None of the inoculated rabbits showed any temperature elevations in the early passages. In later passages, a slightly increased temperature was noticed in some two to three days after inoculation, but temperatures exceeding 40 C. seldom occurred. No other signs of illness were noted. Upon autopsy none of the rabbits showed lesions referable to virus diarrhea.

Calves inoculated with material from rabbits in the early serial passages showed temperatures, leukocyte counts, and other signs of illness similar to that produced by material transferred in calves. Beginning with the thirtieth passage, progressive attenuation occurred, and the only evidence of infection noted with material that had been transferred in rabbits 75 times was a slight leukopenia and a temperature elevation on one day only in the second phase. Calves that were given test virus after inoculation of rabbit-passed material showed no signs of illness and, therefore, had been immunized by the virus in the rabbit material.

Other Animals.—Each animal in the following groups, which consisted of 2 pigs, 2 dogs, 2 cats, 2 goats, 1 sheep, 5 mice, and 2 calves, was inoculated subcutaneously with 1 ml. of a 10 per cent suspension in saline of spleen from an infected calf. Temperatures were taken daily on all animals except the mice. None of them showed any elevation of temperature except the calves, which responded in the usual manner.

DISCUSSION

From 2 cows which showed signs of illness similar to those described for virus diarrhea, infective agents were transferred to calves. In inoculated calves, the course of illness was diphasic, with an initial increase in temperature and a leukopenia two to four days after inoculation, followed by a receding temperature for two or three days and development of a second temperature elevation higher than the first. The two agents procured from natural cases were similar antigenically. Exact comparison studies of these strains could not be made with the original strains since they were no longer available. A comparison of the signs of illness between the animals that furnished the strains and those described in the original publications and of the disease produced in inoculated animals, however, showed a similarity that led to the conclusion that the disease described herein is the same as that named "virus diarrhea."

A diarrhea occurred in about half the calves purchased from dairy farms that responded to inoculation, and occasionally a calf from the disease-free herd developed softer feces. Ulceration of the oral mucosa seen in natural cases rarely occurred in inoculated calves and then only in purchased animals. It would appear that the ulcerations and profuse diarrhea might be a complication and the result of one or more agents acting either secondarily or in combination with VD virus. Failure to produce oral lesions experimentally in the mucosa like those seen in natural cases is not without parallel in rinderpest of cattle. In natural cases, a prominent lesion attributed to rinderpest is ulcerations of the oral mucosa[8] yet in inoculated animals these lesions were almost never seen.[9]

Inoculation of calves purchased from dairy farms showed that about one fourth were immune to VD virus but case histories did not show when this infection had occurred. Almost certainly none of these had developed the oral lesions that lead to clinical recognition of virus diarrhea or they would have been noted. In any investigation for the incidence of virus diarrhea, therefore, reliance must not be placed wholly on oral lesions although these are rather striking and may be the sign that first attracts attention. Also, failure to produce these lesions by inoculation should not be a basis for concluding failure in attempts to procure the initiating infective agent. Recently, Ramsey and Chivers,[10] in reporting on a condition in cattle from Iowa which they called mucosal disease, suggested a similarity between this condition and virus diarrhea and with hyperkeratosis. In transmission attempts, inoculated animals showed an elevated temperature only but no oral lesions and they concluded that they had not explained the etiology. It may be, as our work suggests, that a virus produces widespread infection but requires something else in addition to produce ulcers regularly, yet, without doubt, in this case the VD virus is of primary importance. It is interesting to note that the 2 animals with hyperkeratosis that were inoculated both showed ulcers and died. Further investigation of this relatinoship between the two conditions might prove valuable.

The relationship of virus concentrations to the clinical course of illness is interesting and of importance in making isolations of virus. It appears that after inoculation, viral multiplication begins and reaches a peak about four days after inoculation and then gradually decreases so that eight days after inoculation, and at the height of illness, the blood contains only about ten infectious doses and the spleen 100 infectious doses per gram. When the temperature returns to normal, virus is no longer present in the blood. At this time, the diarrhea and oral lesions are at their height but attempts to isolate the virus might lead to failure.

Identification of an unknown virus strain as "VD virus" is not possible, as yet, by serological means and reliance must be placed on cross-immunity tests in calves. In a test, calves known to be susceptible to VD virus and calves known to be immune must be available. The unknown virus must produce disease in the susceptible calves and fail to do so in the immunized animals and, for final confirmation, the calves that show response to the test virus should become immune to VD virus.

In transfers of VD virus to laboratory animals, it was possible to infect rabbits and to transfer the virus serially in them. In rabbits, the virus showed progressive attenuation and after 75 transfers had so modified in virulence that it can be considered for immunization purposes should a need for a vaccine be shown. The recent

reports on mucosal disease and similar conditions in other parts of the United States that might be virus diarrhea indicate the need to make isolations of the infective agents involved in these conditions and to compare them with VD virus. If the infective agents all prove antigenically similar to VD virus, vaccination to prevent losses might become economically desirable.

SUMMARY

Two strains of virus diarrhea (VD) virus were obtained from 2 cows. They proved antigenically related in cross-immunity tests. Inoculation of calves with this virus intravenously or intranasally produced a diphasic thermal response, leukopenia, general malaise in the second phase and diarrhea in about half the calves. Except in an occasional animal, oral lesions described as typical of virus diarrhea were not seen in experimental calves. One fourth of the calves purchased from dairy farms proved to be immune when inoculated, indicating that the disease may be widespread.

The greatest concentration of virus in the blood and in the spleen was found in the interphase, or four days after inoculation. Smaller amounts of virus were found during the periods of elevated temperatures and none ten days after inoculation when the fever had subsided. Isolations of virus from field cases, therefore, should be attempted before the end of the second phase.

Transfer of virus to rabbits was accomplished. After serial passages in rabbits, the virus became modified. Inoculation of susceptible calves in the seventy-fifth transfer produced a slight decrease in leukocytes and a temperature elevation that lasted for one day only. This modified virus immunized against fully virulent virus.

References

[1] Olafson, P., MacCallum, A. D., and Fox, F. H.: An Apparently New Transmissible Disease of Cattle. Cornell Vet., *36*, (1946): 205.

[2] Olafson, P., and Rickard, C. G.: Further Observations on the Virus Diarrhea (New Transmissible Disease) of Cattle. Cornell Vet., *37*, (1947): 104.

[3] Walker, R. V. L., and Olafson, P.: Failure of Virus Diarrhea of Cattle to Immunize Against Rinderpest. Cornell Vet., *37*, (1947): 107.

[4] Shwartzman, G.: Enhancing Effect of Cortisone Upon Poliomyelitis Infection (Strain MEF1) in Hamsters and Mice. Proc. Soc. Exptl. Biol. and Med., *75*, (1950): 835.

[5] Kilbourne, E. D., and Horsfall, F. L., Jr.: Increased Virus in Eggs Injected with Cortisone. Proc. Soc. Exptl. Biol. and Med., *76*, (1951): 116.

[6] Kilbourne, E. D., and Horsfall, F. L., Jr.: Lethal Infection with Coxsackie Virus of Adult Mice Given Cortisone. Proc. Soc. Exptl. Biol. and Med., *77*, (1951): 135.

[7] Baker, J. A.: Rinderpest Infection in Rabbits. Am. J. Vet. Res., *7*, (1946): 179.

[8] Hutyra, F., Marek, J., and Manninger, R.: Rinderpest. Special Pathology and Therapeutics of the Diseases of Domestic Animals. 5th ed. Alexander Eger, Chicago, *1*, (1946): 256.

[9] Baker, J. A.: Personal observation, 1943-1945.

[10] Ramsey, S. K., and Chivers, W. H.: Mucosal Disease of Cattle. North Am. Vet., *34*, (1953): 629.

Studies on *Neoascaris Vitulorum* III. Further Observations on Inoculation of Calves with Notes on Prenatal Infection

P. G. REFUERZO, D.V.M., and F. S. ALBIS-JIMENEZ, D.V.M.

Manila, Philippine Islands

PREVIOUSLY, in an experiment which precluded the possibility of extraneous infection, Refuerzo *et al.*[1] fed infective eggs of *Neoascaris vitulorum* (Goeze, 1782) to a calf which, when killed after fourteen days, revealed the presence of larvae in the liver and lungs and the conspicuous absence of adult ascarids in the intestinal tract. The parasites from the lungs measured 522.0 μ by 21.6 μ and, save for a slight increase in size, were morphologically indistinguishable from Neoascaris larvae of the same age also obtained from the lungs of infected guinea pigs. Since that time, 20 ascarid-free calves (8 Jerseys, 2 Holstein-Friesians, and 10 Red Sindhis), 1 to 13 days old, had been inoculated to determine the prepatent period of the worm. This is a report of the results of experiments which were conducted along the same methods of approach as those disclosed in the first paper. Briefly, the method consisted of the oral administration of a single dose of 5,000 infective eggs in capsules to each of these calves, putting them in individual stalls with provisions made to forestall reinfection. The feces of these animals were examined up to 120 days following their inoculation and the results are given in table 1.

Due to its biological significance, the authors are also presenting an accumulated data of natural cases of neoascariasis among cattle, carabao (*Bubalus bubalis* Linn.), and Indian buffalo (Bubalus sp.) calves discovered within a few days postpartum. These cases were encountered in routine fecal examinations and treatments of infected cattle in stock farms in the Philippine Islands since 1941. The calves began to pass unsegmented eggs of Neoascaris in their dejecta when only 14 to 42 days old. However, only animals that showed parasitism within thirty days of birth were selected as subjects for this study. To date, there are 35 such cases given in tables 2 and 3.

DISCUSSION

The feeding of 5,000 infective eggs to each of 20 calves resulted in "negative" infection in the sense that no adult worms were produced in periods of 110 to 120 days (table 1). Using big doses of infective eggs, Brumpt [2] and Herlich and Porter [3] also failed to establish infection in 3 calves and 7 calves, respectively. The latter workers fed their animals with single or multiple doses of 1,000 to 108,000 eggs with negative results, but they succeeded in producing prenatal infection in 1 of twin calves by feeding the dam 41,800 infective ova in 17

TABLE 1—Results of Feeding Infective Neoascaris Vitulorum Ova to Young Calves

Calf (No.)	Age when infected (days)	Infective ova in inoculum (No.)	Results days feces remained negative * (No.)
R.S. 28	4	About 5,000	110
R.S. 13	6	About 5,000	113
R.S. 34	4	About 5,000	110
R.S. 19	5	About 5,000	110
R.S. 29	3	About 5,000	117
R.S. 21	2	About 5,000	110
R.S. 42	7	About 5,000	110
R.S. 23	13	About 5,000	115
R.S. 26	8	About 5,000	110
J. 43	1	About 5,000	116
R.S. 84	4	About 5,000	110
J. 29	4	About 5,000	110
J. 1	5	About 5,000	120
J. 47	5	About 5,000	112
J. 35	8	About 5,000	110
J. 45	13	About 5,000	111
J. 19	11	About 5,000	110
H.F. 131	6	About 5,000	110
H.F. 1391	7	About 5,000	113
J. 15	3	About 5,000	110

* Feces examined every other day beginning one week after feeding the ova; R.S.—Red Sindhi; J.—Jersey; H.F.—Holstein-Friesian.

Dr. Refuerzo is chief of the Parasitic Diseases Division, Bureau of Animal Industry, Philippine Islands and a member of the faculty of the College of Veterinary Medicine, University of the Philippines, Manila.

Dr. Albis-Jimenez is veterinary helminthologist, Bureau of Animal Industry, Philippine Islands.

The authors acknowledge their indebtedness to Drs. William D. Lindquist and Irving P. Delappe, professor and assistant professor in parasitology, respectively, in the School of Veterinary Medicine, Michigan State College, East Lansing, for their many helpful suggestions, and to the personnel of the dairy project, Alabang Stock Farm, Rizal, Philippine Islands, for their cooperation during the progress of the experiment.

doses from impregnation until parturition. This result is significant because it reveals one of the modes of infection with *N. vitulorum*.

TABLE 2—Natural Cases of Neoascaris Among Cattle Calves Discovered Within a Few Days Postpartum at the Alabang Stock Farm, Rizal, Philippine Islands

Calf (No.)	Age when positive (days)	Results, treatment, and remarks
R.S. 47	24	3 female, 2 male worms expelled
R.S. 22	21	2 males, 2 females voided
R.S. 16	20	2 females (1 immature), 3 males voided
R.S. 27	21	4 females, 1 male expelled
R.S. 18	14	1 male, 2 females expelled
R.S. 49	20	1 male, 1 female expelled
R.S. 57	24	5 females, 3 males expelled
R.S. S-6	24	4 females, in diarrheic stool
R.S. S-32	19	Adult ascarids voided without deworming
Nellore S-5	29	Feces positive for eggs 12-28-46; died 1-2-47, many ascarids in intestine
R.S. S-1	30	2 females, 1 male voided
R.S. S-33	26	1 female, 1 immature male voided
R.S. S-18	27	Worms in diarrheic stool
G. S-10	27	3 females, 1 male voided
G. S-3	24	1 female, 2 males voided
G. 31	22	2 males, 1 female expelled
G. 24	23	Adult worms in stool.
G. 22	28	1 female worm voided

R.S.—Red Sindhi; G.—Grade

Perusal of tables 2 and 3 shows that within fourteen to thirty days of birth cattle, carabao, and Indian buffalo calves voided eggs of the ascarid in their stools and treatment resulted in the expulsion of the worms. The prepatent period or the complete development of this helminth in its definitive host is still unknown, and the early appearance of eggs in the calves' dejecta suggests either (1) an exceedingly rapid growth of *N. vitulorum* inside its host (granting that the calves ingested infective eggs immediately after birth) or (2) an antepartum development of the parasites which the calves acquired *in utero*. The first supposition is hardly tenable because no known member of the family Ascarididae, occurring in livestock, has ever been known to attain sexual maturity within this length of time (14 to 30 days). Furthermore, it was found that fourteen days after the oral administration of infective ova to a calf the parasites were only in their larval stages in the liver and lungs and no mature ascarids were seen in the intestinal tract.[1] This worm evidently can not become oviferous and lay eggs within fourteen days, and it is unlikely that it can attain this stage within 30 days, judging from its minute size (522 μ by 21.6 μ) or growth after fourteen days of infection.

The life cycle of this nematode has been assumed by Monnig,[4] by Morgan and Hawkins,[5] by Mayhew,[6] by Schwartz,[11] and by others, to be similar to that of *Ascaris lumbricoides* which has a prepatent period of eight to nine weeks although Martin[7] found it to mature a little earlier (49 days).

In view of the above, it would not be easy to explain the early appearance of eggs in the stools of the 35 naturally infected calves unless it is ascribed to an infection, or to an antepartum development of the parasites, which the calves acquired before birth. Prenatal infection was suggested by Boulenger,[8] Griffiths,[9] Macfie,[10] Schwartz,[11] Sprent,[12] Vaidyanathan,[13] and Keith,[14] and this mode of transmission was proved experimentally by Herlich and Porter[3] who established prenatal infection in 1 of twin calves by feeding the dam with huge doses of infective ova.

Basing their contention on their, and other investigators',[2,3] consistent failure to produce infection with adult worms in several calves by the oral administration of big doses of infective ova, and on the frequency with which congenital neoascariasis is met with in certain areas in the Philippine Islands, the authors are inclined to believe that prenatal infection is the usual mode of spread in *N. vitulorum*, as sug-

TABLE 3—Natural Cases of Neoascariasis Among Carabao and Indian Buffalo Calves Discovered Within a Few Days Postpartum at the Swiss Dairy Farm, and at the Alabang Stock Farm, Rizal, Philippine Islands

Calf (No.)	Age when positive (days)	Results, treatment, and remarks
Carabao 94	29	4 female, 3 male worms expelled on treatment
Carabao 95	23	2 females, 2 males voided
Carabao 13	22	1 female, 2 males expelled
Carabao 10	20	1 female, 2 males expelled
Carabao 4	30	4 females, 1 male expelled
Carabao 25	27	1 female, 3 males expelled
Carabao 39	26	1 female, 1 male expelled
Carabao 44	25	Heavily parasitized, passed balls of dead worms (134 females, 44 males); died 11-5-42
Carabao 101	24	1 female voided
Carabao 9	27	3 females, 2 males expelled
Carabao 102	24	5 females, 7 males expelled
Carabao 41	22	3 females, 6 males voided, feces still positive 12-21-42
Carabao 36	24	2 females, 2 males (1 immature) expelled
Carabao 18	26	1 female, 1 male voided
Carabao 27	27	3 females, 7 males expelled
Buffalo 16	28	Passed several worms in dejecta without treatment
Buffalo 90	28	5 females, 2 males expelled

gested earlier by Sprent.[15] However, they are of the opinion that postnatal infection also occurs because calves previously treated will again become infected (Monnig, personal communication). Moreover, the occasional findings in the Philippine Islands by the senior author of very light infections with ovigerous Neoascaris in adult cattle, which were previously free from this parasite, lend further support to this latter view.

How prenatal neoascariasis is bruoght about is still conjectural. According to Sprent,[15] the larvae of certain species of "ascaris parasites" exhibit two kinds of migratory behavior inside their hosts, namely, tracheal and somatic, depending on the route of larval migration following the ingestion of infective eggs. He found that *Toxocara canis* of dogs follows a somatic type of migration in white mice and suggested that *N. vitulorum* may exhibit a similar migratory behavior. He thinks that "with both these parasites prenatal infection appears to be the usual mode of spread. If an exclusively somatic type of migration occurred in the definitive host, as was observed in the mouse infected with *T. canis*, larvae would not reach the intestinal lumen of the animal ingesting the eggs but would, among other tissues, be distributed to the placenta, whence they could reach the fetal liver and lungs. In this way, prenatal infection would become the only mode of infection."

Do the larvae of this ascarid follow the somatic, the tracheal, or both types of migration inside the host? The results of the feeding experiments of Brumpt,[2] of Schwartz,[16] of Herlich,[17] and of Refuerzo *et al.*[1] involving unnatural and natural hosts show at least that the larvae follow the lung journey or the tracheal type of migration. On the other hand, it would be difficult to explain prenatal infection without attributing it to the somatic migration of the larvae. Further studies along this line are necessary in order to clear this point.

SUMMARY

Twenty calves representing three breeds of various ages from 1 to 13 days old were each fed a single dose of 5,000 infective Neoascaris ova with "negative" results. No eggs of the ascarid were found in the feces of these animals up to 120 days. Thirty-five cases of neoascariasis among cattle, carabao (*Bubalus bubalis* Linn.), and Indian buffalo (Bubalus sp.) calves discovered within fourteen to thirty days after birth are presented. The early appearance of eggs or of ovigerous worms in these calves is attributed to prenatal infection which is believed to be the usual, but not the only, mode of transmission in *Neoascaris vitulorum*. The mechanism of prenatal infection as well as the migratory behavior of the larvae of this ascarid in its host are discussed.

References

[1] Refuerzo, P. G., Albis-Jimenez, F. S., and Cerna, G. de la: Studies on *Neoascaris vitulorum* (Goeze, 1782). I. Observations on the Morphology of the Ova and on their Development to the Infective Stage. With Notes on Feeding Experiments. Philippine J. Anim. Indust., *13*, (1952): 15-23.

[2] Brumpt, E.: Precis de Parasitologie. 5th ed. Masson et Cie, Paris, (1936): 865.

[3] Herlich, H., and Porter, D. A.: Prenatal Infection of a Calf with the Nematode *Neoascaris Vitulorum*. (Abstr.) J. Parasitol., *39*, (1953): 33-34.

[4] Monnig, H. O.: Veterinary Helminthology and Entomology. 3rd ed. William Wood and Co., Baltimore. 1949.

[5] Morgan, B. B., and Hawkins, P. A.: Veterinary Helminthology. Burgess Publishing Co., Minneapolis. 1953.

[6] Mayhew, R. L.: The Parasites and Parasitic Diseases of Cattle. Louisiana Bull. 428, (1953): 1-46.

[7] Martin, H. M.: Studies on the *Ascaris lumbricoides*. Nebraska Exptl. Sta. Res. Bull. 37, (1926): 1-78.

[8] Boulenger, C. L.: On *Ascaris Vitulorum* Goeze. Parasitology, *14*, (1922): 87-91.

[9] Griffiths, J. A.: Prenatal Infection with Parasitic Worms. Vet. J., *78*, (1922): 478-481.

[10] Macfie, J. W. S.: The Ascaris of Cattle. Ann. Trop. Med. and Parasitol., *16*, (1922): 311-313.

[11] Schwartz, B.: Occurrence of Ascaris in Cattle in the United States. North Am. Vet., *6*, (1925): 24-30.

[12] Sprent, J. F. A.: Some Observations on the Incidence of Bovine Helminths in Plateau Province, Northern Nigeria. Vet. J., *102*, (1946): 36-40.

[13] Vaidyanathan, S. N.: *Ascaris Vitulorum*—Prenatal Infection in Calves. Indian Vet. J., *26*, (1949): 228-230.

[14] Keith, R. K.: The Occurrence of *Ascaris Vitulorum* Geoze, 1782, in Calves in Australia. Austral. Vet. J., *27*, (1951): 129-131.

[15] Sprent, J. F. A.: On the Migratory Behavior of the Larvae of Various Ascaris Species in White Mice. I. Distribution of Larvae in Tissues. J. Infect. Dis., *90*, (1952): 165-176.

[16] Schwartz, B.: Observations on the Life History of *Ascaris Vitulorum*, a Parasite of Bovines in the Philippine Islands. Preliminary paper. Philippine J. Sci., *20*, (1922): 663-669.

[17] Herlich, H.: On the Migratory Behavior of the Larvae of *Neoascaris vitulorum* (Goeze, 1782). Travassos, 1927, in White Mice. Proc. Helminthol. Soc. Washington, *20*, (1953): 124-126.

Correction

With reference to "Studies on *Neoascaris Vitulorum*. II. The Resistance of the Ova to Certain Chemical Agents and Physical Factors Under Tropical Conditions" by Doctors P. G. Refuerzo and F. S. Albis-Jimenez, published in the July issue of the AMERICAN JOURNAL OF VETERINARY RESEARCH (pp. 440-443), Dr. Refuerzo requests that, wherever in the article "embryonating" and "nonembryonating" are used, these words be changed to *embryonated* and *nonembryonated*.

Disturbances in Acid-Base Balance and Electrolyte in Calf Diarrhea and Their Treatment

A Report of Eighteen Cases

B. J. McSHERRY, D.V.M., and I. GRINYER

Guelph, Ontario

DIARRHEA, a common ailment of young calves, causes many deaths. For this reason, it is of importance both to the livestock industry and to the veterinary profession. Treatment is mainly directed toward identifying and subsequently eliminating the causative organisms with the use of antibiotics and chemotherapeutic agents. Many animals fail to respond to this treatment, in some cases because of increased resistance of the pathogens to these agents. However, in other instances this is not the reason. It is apparent, therefore, that a more searching approach to therapy must be considered. Perhaps the focus of attention should be diverted from the obvious symptoms to the changes produced on the body as a whole. To understand the changes, a knowledge of the physiological disturbances occurring in this condition is necessary.

Disturbances in electrolyte and in acid-base balance are seen in many pathological conditions and it is to be expected that appreciable changes would occur in diarrhea where there is loss of water and electrolyte in the feces. Blaxter and Wood [2] have recently shown that in calf diarrhea there is an increase in weight of feces up to 40 per cent which is due mainly to increased excretion of water; this is accompanied by considerable loss of calcium, magnesium, phosphorus, sodium, and potassium. Extensive loss of intracellular potassium in infant diarrhea has been reported by Darrow.[4] Darrow *et al.*[6] contend that loss of intracellular potassium is accompanied by the cellular entrance of sodium, either during dehydration due to diarrhea or during the treatment phase if potassium is not included in repair solutions. Gamble [9] points out that chloride and sodium are particularly exposed to loss in diarrheal stools as they are components of the gastrointestinal secretions. Bland [1] states that infants have relatively more water in their bodies than adults, the greater proportion of it being in the extracellular fluid, and that electrolyte deficits will occur about seven times more readily in a child than in an adult. Hand and Leininger [10] state that a 7-kg. infant has 1,400 cc. of extracellular fluid and the daily intake and output is 700 cc., or 50 per cent of his reservoir. This is compared to an adult with 14,000 cc. of extracellular fluid and a daily water exchange of 2,000 cc., or 14 per cent of his reservoir. Thus, the susceptibility of a young animal to rapid dehydration is much greater than that of an adult.

It is not within the scope of this paper to discuss in detail the chemistry of acid-base balance and of body electrolyte; however, some of the more pertinent facts of importance to this study are outlined.

The bicarbonate-carbonic acid system is one of the most important buffer systems in the body, and changes in this system reflect changes of the acid-base balance of the body as a whole. Retention of acids or bases is reflected by a change in the serum bicarbonate content. The bicarbonate system is a mixture of carbonic acid (H_2CO_3) and bicarbonate ($BHCO_3$), the base being mainly sodium or potassium. This supply of base which is available to neutralize acids is termed the "alkali reserve." The total amount of carbon dioxide present as carbonic acid and bicarbonate is readily measured by gas analysis. Normally, the ratio of carbonic acid to bicarbonate is 1:20 and most pathological changes in acid base can be considered from the standpoint of changes in this ratio. If the pH of the serum is known, the carbonic acid-bicarbonate ratio can be determined by use of the Henderson-Hasselbalch equation $\left(\mathrm{pH} = 6.1 + \log \frac{(\mathrm{BHCO_3})}{(\mathrm{H_2CO_3})}\right)$. Thus, at a pH of 7.4 the ratio is 1:20, at a pH of 7.2 it is 1:12, while at a pH of 7.1 it declines to 1:10. It is difficult to set a definite pH below which a condition of acidosis may be said to prevail in the body. However, it can be said that an apparent uncompensated acidosis is present if the pH is lower than 7.3. Cantarow and Trumper [3] mention that, clinically, the most common cause of acidosis is a primary alkali deficit which may

From the Ontario Veterinary College, Guelph, Ontario. Mr. Grinyer is an Associate of the Institute of Medical Laboratory Technology.

The authors are indebted to Dr. F. D. Horney of the Department of Medicine and Surgery, Ontario Veterinary College, for his aid in the clinical examination and treatment of the cases admitted to the college clinic.

occur when there is excessive loss of base from the body. Sodium forms nine tenths of the base in the extracellular fluid while potassium is the main base in the intracellular fluid. Chlorides form two thirds of the anions in the extracellular fluid and together with bicarbonate ion constitute 85 per cent of the anions in this portion. Sodium is normally present in the cells in small concentrations but, under abnormal conditions, may enter the cells in increased amounts, replacing potassium. When this occurs, the potassium leaves the cells and enters the serum and, if renal function is normal, the excess is excreted in the urine. While this is taking place it is possible, as Darrow [5] has shown, to have high serum potassium levels even though there is an over-all deficiency of potassium in the body.

In dehydration due to diarrhea or other causes, it is considered imperative to replace the lost fluid with parenterally administered solutions. The sole use of 0.9 per cent sodium chloride for this purpose is not without danger as it contains an excess of chloride ions and is lacking in other ions usually depleted in such conditions. Fox *et al.*[8] have shown that the administration of 0.9 per cent sodium chloride to dogs requiring fluid therapy produced a decrease in serum potassium levels. They suggest using a balanced electrolyte solution approximating normal serum values with the exception of potassium and bicarbonate which are doubled in amount. The bicarbonate ion is provided in the form of rapidly metabolized precursors, acetate, and citrate. Mudge *et al.*[14] have shown that sodium acetate when used as a source of fixed base in dogs and man rapidly increases the plasma carbon dioxide content.

TABLE 1—Chemical Composition of Balanced Electrolyte Solution

	Grams per liter	Milliequivalents per liter				
		Na	Cl	K	Ca	Mg
NaCl	5.5	95	95	....	....	....
$CaCl_2$	0.3	....	5	....	5	....
$MgCl_2 6H_2O$	0.3	....	3	....	....	3
$NaC_2H_3O_2 3H_2O$ (Sodium acetate)	5.0	37	....	....	....	....
$KC_2H_3O_2$ (Potassium acetate)	1.0	....	....	10	....	....
$Na_3C_6O_7 2H_2O$ (Sodium citrate)	0.8	8	....	....	....	....
Total		140	103	10	5	3

Sodium citrate can be omitted if the sodium acetate is increased to 6.1 Gm.

The purpose of this present study is twofold—first to observe and record changes in the acid-base level and electrolyte in the serums of calves with diarrhea; second, to observe the effect of a parenterally administered electrolyte solution on these changes.

TABLE 2—A Comparison of the Constituents of Calf Serum, 0.9 per Cent NaCl and Balanced Electrolyte Solution

	Milliequivalents per liter					
	Na	Cl	K	Ca	Mg	HCO_3
Calf serum	144	103	5	5	3	30
0.9 % NaCl	154	154	....	....	....	....
Balanced electrolyte solution	144	103	10	5	3	....

Materials and Methods

The method of collecting the blood samples and the technique of serum analysis used in this study are the same as those described by the authors [13] in a previous paper. In addition, blood sugar levels and hematocrit values were included in the present study. Blood sugar was measured on 0.05 cc. of whole blood using the method described by King.[12] The hematocrit values were obtained by the use of the Wintrobe hematocrit tube centrifuged at 2,500 r.p.m. for one-half hour. In preparation for the present study, blood sugar levels were determined on 20 normal calves between the ages of 4 days to 10 weeks. The results showed the mean to be 76.8 mg. per hundred cubic centimeters with a standard deviation of plus-minus 12.8 mg.

The animals selected for the present study were calves, available to the college clinic, showing clinical evidence of diarrhea. Many had been treated previously either by veterinarians or by the owners. Where possible, successive samples were taken until the animal either recovered or died.

A balanced electrolyte solution was prepared and used for parenteral therapy during this study. Table 1 shows the chemicals used in preparing this solution and indicates the ions obtained from each. Table 2 compares this solution with calf serum and 0.9 per cent sodium chloride.

Originally, it was planned to give the solution intravenously and a portion of the bicarbonate ion precursor was added as citrate which by also acting as an anticoagulant would help to prevent clotting in the needle. However, in practice the solution was given intraperitoneally thus obviating the necessity of the citrate. This is obviously the route of choice for administering the solution where speed and ease of handling is a consideration.

It is possible to calculate from the weight of the patient and the bicarbonate level of the serum the approximate volume of the solution required to repair the electrolyte deficit.[10] Fox *et al.*[8] have used 50 to 150 cc. per kilogram of body weight of a

similar solution to restore normal hydration and electrolyte values in pediatric patients. In the present study, the volume of electrolyte solution given was varied according to the clinical appearance of the animal.

CASE HISTORIES

Cases 1, 2, and 3, were Shorthorn calves, 28 days old, in a well-cared-for herd; diarrhea had been present for less than eight hours when the Ambulatory Clinic of the Ontario Veterinary College was consulted. The calves were bright, active, and able to eat, but all had elevated temperatures and voluminous yellow stools. Antibiotic therapy was given immediately and on day 2* the feces were normal.

Case 4 was a Jersey calf, 11 days old, on a farm where sanitation was poor and diarrhea common; diarrhea was evident at least one day before the first blood sample was taken. On day 1, the animal was dull and lethargic but able to walk and feed; on day 2, the diarrhea had moderated, and on day 6 the animal was clinically normal.

Case 5, a Jersey calf 11 days old, was kept in the same pen as case 4. Diarrhea had been evident for two days. On day 1, the animal was weak and incoordinate but able to walk and feed; on day 2, the diarrhea and physical condition was worse; on day 6, the feces were firm and the calf was clinically normal.

Case 6, a Jersey calf 11 days old, was kept in the same pen as cases 4 and 5. The animal had had diarrhea several days previous to day 1, but at no time during the study was diarrhea present. Cases 4, 5, and 6 were treated by the owner with chemotherapeutic agents supplied by a veterinarian.

Case 7, a Jersey calf 10 days old, was kept on a farm where calves were raised for veal. Diarrhea was endemic and losses in the past two years were over 50 per cent. Sanitary conditions were appalling. All forms of treatment had failed to affect the course of the disease on previous cases and this animal remained untreated. Watery diarrhea had been evident for three to four days. On day 1, it was weak but able to nurse; on day 7, the clinical condition was normal.

Case 8, a Holstein-Friesian calf 14 days old, was admitted to the clinic with a moderate diarrhea which had not responded adequately to several days of treatment by a veterinarian. On day 1, the animal was weak and remained on its feet with difficulty. One liter of electrolyte solution was administered intraperitoneally; on day 2, the animal had recovered sufficiently to be discharged.

Case 9 was a Holstein-Friesian calf 10 days old. On day 1, the animal was dull and lethargic, and diarrhea was profuse; on day 2, the animal was unable to rise. Diarrhea was less evident on day 3, and on day 4, the feces were normal and the clinical appearance much improved. Antibiotic therapy was started twenty-four hours before day 1 and was repeated on days 1, 2, and 3.

Case 10, a Holstein-Friesian calf 25 days old, had had diarrhea for three days previous to admittance to the clinic. The animal was depressed, with evidence of early pneumonia and moderate diarrhea. One liter of electrolyte solution was administered on day 1 and two liters on day 2. Penicillin was given on days 2 and 3. On day 2, diarrhea was less evident; on day 4 the feces were almost normal.

Case 11, a Shorthorn calf 8 days old, had had diarrhea for an undetermined time prior to admittance to the clinic. Antibiotic therapy given by a practicing veterinarian had failed to relieve the condition. The calf was comatose, with a cold clammy mouth, shallow breathing, and glazed sunken eyes. Two liters of electrolyte solution were administered on day 1. On the morning of day 2 the animal was bright, able to walk, and drank milk eagerly; the temperature was 102.5 F.; the feces were soft but not diarrheic. Two liters of electrolyte solution were administered on day 2. Recovery was uneventful. An interesting feature was the calf's craving for the electrolyte solution. It would not drink tap water and preferred the electrolyte solution to milk, licking the container long after it was empty.

Case 12, a Jersey calf 10 days old, was kept in the same pen as case 7. Profuse watery diarrhea was first evident on day 1, and though moderating, was still present on day 4. The calf was weak and incoordinate but was able to walk and feed. It was given 500 cc. of electrolyte solution on day 4 and again on day 5. On day 6, the clinical condition had improved, the animal was stronger, and the feces almost normal.

Case 13, a Shorthorn calf 28 days old, was kept on the same farm as cases 1 to 3. Diarrhea had been present for five days, and the animal was weak and unable to rise on day 1. On the day prior to this, it was not possible to get a blood sample as the jugular vein could not be raised. On day 2, the animal was able to stand and nurse but was extremely weak; on day 3, it could walk but was incoordinate. When the final sample was taken on day 10, the clinical condition was normal. Antibiotic therapy was given throughout the course of the disease.

Case 14, a Jersey calf 7 days old, was kept in the same pen as case 7. On day 1, watery diarrhea was evident, the clinical condition was fair, and the animal was able to feed. On day 6, the diarrhea was more severe, and the feces were blood stained; 500 cc. of electrolye solution was given. On day 7, the animal, while able to stand, was very weak, and diarrhea was profuse. At this time it was transported to the clinic. Between days 7 and 10, 4½ liters of electrolyte solution and 3 Gm. of streptomycin were given. There was progressive improvement until on day 10 the animal appeared normal. On day 15, hematological studies showed the animal to be anemic.

* In the case histories, day 1 refers to the day the first blood sample was taken. Subsequent days are measured in 24-hour periods from this time.

Case 15, a Shorthorn calf 21 days old, was admitted to the clinic in a moribund state. Two weeks previously diarrhea responded to treatment with streptomycin but recurred when this therapy was discontinued. On day 1, the temperature was 97 F., the mouth was cold and clammy, and the animal was comatose. Two and one-half liters of electrolyte solution containing 5 per cent glucose was administered. On day 2, milk was given by stomach tube and 1.5 liters of the electrolyte solution containing 5 per cent glucose was administered. On the evening of day 3, the animal was much stronger, drank eagerly from a bottle, and the temperature was 102 F. On the morning of day 4, it was again in a coma and died one-half hour after the last sample was taken.

Case 16, a Jersey calf 14 days old, was kept in the same pen as case 7. Diarrhea had been evident for several days. The animal was in such a deep coma that the owner thought it had been dead for several hours. However slight breathing could be detected, and no difficulty was encountered in getting blood to flow from the jugular vein. The animal died one-half hour later.

Case 17, a Shorthorn calf 20 days old, was admitted to the clinic in a moribund state following three days of severe diarrhea; antibiotic therapy had failed to alleviate the condition. Three liters of electrolyte solution was administered, but the animal died during the night of day 1.

Case 18, a Holstein-Friesian heifer 3 months old, was admitted to the clinic after eight days of diarrhea; it was weak and dehydrated. Samples were taken on day 1 following which 6 per cent dextrose in normal saline was given intravenously. This treatment was repeated on day 2. When samples were taken on day 3, saline and dextrose had not been administered for twenty-four hours. Two liters of electrolyte solution were given on day 3. The animal died during the night of day 3.

Results

Table 3 outlines in detail the results of analysis in the 18 cases.

Salient Findings.—In cases 1, 2, and 3, the serum potassiums and sodiums were elevated despite the short duration of the disease.

In case 4, the serum bicarbonate was low while diarrhea was evident. Serum chloride was elevated and sodium was subnormal in the course of the disease. Following recovery, the serum bicarbonate rose above normal.

In case 5, the same trend as in case 4 was evident. However, the serum bicarbonate was lower, and at one period the pH was below normal. This is in keeping with the clinical findings which were more severe.

In case 6, the serum bicarbonate was elevated as in cases 4 and 5 following recovery.

In case 7, the serum bicarbonate and chloride were subnormal and the potassium was in the low normal range while the diarrhea was evident. During this time, serum sodium was elevated. All values were within the normal range following clinical recovery.

In case 8, the serum bicarbonate was moderately low and the serum potassium markedly low before treatment. Both were approaching normal within twenty-four hours of treatment.

In case 9, the electrolyte changes closely paralleled the clinical condition. The serum bicarbonate, chloride, and calcium were low, and serum potassium was high when symptoms were severe, returning to normal as the clinical condition improved.

In case 10, the serum bicarbonate, calcium, and potassium were low prior to treatment. Within twenty-four hours of treatment, all were returning to normal.

In case 11, the serum bicarbonate, calcium, and sodium were subnormal and the serum potassium and inorganic phosphorus (12.5 mg./100 cc.) were increased when the clinical condition was severe. Within forty-eight hours of treatment with the repair solution, the electrolyte pattern and clinical condition were normal.

In case 12, the serum electrolyte was normal on the first day diarrhea was evident. Three days later, the serum bicarbonate and pH were subnormal and the serum sodium and potassium elevated. The serum sodium remained elevated following treatment with electrolyte solution the others returning to normal.

In case 13, the serum bicarbonate and pH were subnormal while the animal was clinically ill. The serum potassium became subnormal in the later stages of the disease. Low serum chlorides were present following clinical recovery.

In case 14, the changes in electrolyte followed a rather definite pattern. The serum bicarbonate and pH became progressively lower while diarrhea was evident, returning slowly to normal as the clinical condition improved. The serum sodium was elevated early in the disease but fell sharply at the midpoint. At this point the serum chlorides, which had been high from the

TABLE 3—Blood Chemical Values and Hematocrit Levels in 18 Cases of Calf Diarrhea

		Milliequivalents per liter						Mg./100 cc.	Vol. packed red cells (%)
		pH	HCO_3	Cl	Na	K	Ca	Sugar	(Hematocrit)
Normal values[12]		7.30–7.54	31	103	142	5.2	5.0	76	
Case (No.)	Day*								
1	1	7.50	32.2	99	158	6.4	4.7	72	32
	2	7.50	30.5	100	154	6.9	4.8	134	31
2	1	7.40	31.5	104	152	7.2	5.1	127	37
3	1	7.42	34.5	107	159	7.2	5.1	103	32
4	1	7.35	22.0	116	151	5.5	5.6	72	31
	2	7.35	22.7	106	132	5.1	5.6	88	29
	6	7.50	36.7	101	136	5.0	5.3	134	29
	8	7.45	34.9	99	151	6.5	4.8	152	30
5	1	7.30	17.8	113	145	5.8	5.6	70	26
	2	7.27	15.7	109	152	5.8	5.4	127	29
	6	7.50	37.3	102	137	4.8	5.3	95	27
	8	7.50	35.8	104	145	5.5	5.0	228	24
6	1	7.45	37.0	100	152	5.4	5.2	99	22
	5	7.42	33.3	102	150	4.7	5.0	134	30
	7	7.47	33.0	103	149	5.5	4.4	163	25
7	1	7.40	22.7	92	166	4.5	5.7	33	29
	7	7.45	31.3	103	144	4.8	5.0	114	21
8	1	7.40	23.0	98	149	3.7	4.8	58	44
	2	7.47	29.0	100	151	4.4	4.8	....	40
9	1	7.42	27.6	87	139	6.9	4.4	91	42
	2	7.30	22.3	84	144	9.0	3.2	84	43
	3	7.32	22.7	91	139	7.7	4.7	86	34
	4	7.32	24.8	100	140	7.3	4.8	79	32
	7	7.35	27.3	103	144	6.0	4.9	91	32
10	1	7.37	19.9	107	155	4.3	4.0	76	25
	2	7.40	25.5	104	151	4.7	4.1	63	24
	3	7.42	29.6	103	152	4.8	4.3	65	19
	4	7.42	29.9	103	151	4.9	5.3	51	19
11	1	....	18.0	.....	130	11.0	2.6	114	....
	2	....	20.0	.....	133	12.4	3.0	....	....
	3	....	27.3	.....	148	5.0	4.6	109	....
12	1	7.45	26.6	105	145	5.7	4.8	120	36
	4	7.12	17.8	100	161	7.0	5.1	84	30
	5	7.32	21.0	98	168	5.8	5.3	120	28
	6	7.37	27.0	100	161	5.5	5.6	124	28
13	1	7.20	14.1	104	147	5.1	5.0	87	27
	2	7.20	17.0	105	148	4.3	4.5	87	29
	4	7.37	20.3	107	145	3.8	4.7	114	26
	10	7.42	28.9	91	152	5.8	5.0	106	24
14	1	7.45	30.3	108	145	6.0	4.9	95	27
	2	7.37	28.2	108	142	6.2	4.6	134	29
	5	7.35	29.0	106	160	6.4	4.6	84	29
	6	7.05	10.6	105	160	6.0	5.0	114	28
	7	7.0	8.1	106	139	5.6	5.0	126	28
	8	7.0	9.2	116	119	6.4	5.6	60	28
	9	7.20	11.2	116	138	5.0	4.5	81	21
	9.5	7.27	12.1	113	142	5.7	4.6	84	20
	10	7.27	14.5	102	157	4.6	5.8	100	20
	12	7.25	18.0	103	145	5.3	5.1	87	17
	13	7.30	22.8	103	144	4.4	5.0	88	17
	15	7.40	30.5	101	137	4.8	4.6	91	16
15d	1	7.27	12.2	104	148	5.2	5.0	33	29
	2	7.32	31.4	109	146	5.2	5.4	228	33
	3	7.42	27.9	106	154	4.8	5.6	35	28
	4	7.42	32.8	103	154	5.4	5.0	<10	25
16d	1	7.17	15.7	100	131	4.5	5.0	<10	31
17d	1	<7.0	4.65	124	162	6.8	5.3	82	....
18d	1	7.32	21.7	92	173	3.8	5.8	....	....
	3	7.12	17.5	72	176	3.7	5.6	450	45

* Day 1 refers to the first day on which samples were taken for analysis and the succeeding days represent 24-hr. periods from this time.

Inorganic phosphorus values were normal in all cases with the exception of 11 and 18 where they were elevated as mentioned in the text.

d—Calf died.

beginning, rose markedly. The serum potassium was high in the early stages becoming subnormal later.

In case 15, the serum bicarbonate, pH, and whole blood sugar were all low on admittance but rapidly returned to normal after treatment with electrolyte solution containing glucose. Despite the definite clinical improvement and the return of appetite, the blood sugar dropped and was less than 10 mg. per 100 cc. just prior to death.

In case 16, the serum bicarbonate, sodium, and pH were low and, as in case 15, the blood sugar was less that 10 mg. per 100 cc.

In case 17, the state of electrolyte and pH differed from the previous cases chiefly in the magnitude of the deficit of bicarbonate and sodium and the very low pH.

In case 18, the serum bicarbonate, pH, chlorides, and potassium were low and the sodium elevated on admittance. These changes were accentuated on day 3, and at this time inorganic phosphorus (15 mg./100 cc.) and blood sugar levels were elevated.

Discussion

The most constant finding in the 18 cases was the decrease in the serum bicarbonate levels accompanied by a lowering of the serum pH. In 8 of the cases the pH was below 7.3 at some stage of the disease and a condition of uncompensated acidosis prevailed. Compensation in most cases was maintained until the bicarbonate declined to the region of 20 mEq. per liter. Presumably the acidosis is due either to excess formation and retention of acids or to the loss of base. Many workers in the human field [1, 3, 4, 9, 10] consider that the metabolic acidosis accompanying diarrhea is due mainly to loss of base. In a series of 6 cases of diarrhea studied by Darrow,[4] the serum sodium values were all below normal. In the studies on calves reported here, the serum sodium levels were not uniformly altered but increased greatly in some cases and decreased in others. The low sodium is to be expected if this element is lost in quantity from the body during the course of the disease, but the increase is more difficult to explain. Darrow and Pratt,[7] in discussing infantile diarrhea, state that there is considerable variation in the absolute and relative losses of sodium, potassium, and chloride as the serums may be hypotonic or hypertonic and that the low concentration of bicarbonate in infant diarrhea indicates relative deficiency of sodium in extracellular fluids.

A feature of the cases studied is the variation from normal in the serum potassium levels, these being increased in many instances and decreased in others. In the present study, it would appear that in most instances the high potassium levels represent potassium leaving the cells in the early stages of the disease while the low levels indicate the end result of this process. Harrison [11] states that one of the most common clinical signs of cellular potassium deficit is weakness and stupor and it is noteworthy that these were prominent symptoms in the cases presented in the present paper. Darrow [4] mentions finding levels of potassium as high as 10 to 12 mEq. per liter in moribund diarrheic patients and this corresponds to the findings in case 8.

The variation of serum chlorides from normal was considerable in some of the cases. It is known that prolonged loss of chloride in body excretions will lead to hypochloremia. Also, it is an accepted fact that in acidosis when the bicarbonate falls the chlorides tend to rise to compensate for this. In the calves, the low chlorides are probably due to excessive loss, the high chlorides to the compensatory mechanism.

In many of the calves, the calcium levels were in the low normal range, while in 3 (cases 9, 10, 11) the values were subnormal. The explanation for this is possibly the excessive loss of this ion in the feces.

The glucose levels are of interest in that in at least 3 cases (7, 15, 16) they were below normal, and in 2 of these (15, 16) glucose was less than 10 mg. per 100 cc. In some of the other cases, high sugar levels were present following recovery.

Since the hematocrit levels were determined on successive days, they give an indication of the extent of dehydration. In some but not all cases, the hematocrit levels declined as the clinical condition improved.

Summary

Serum pH, bicarbonate, chloride, sodium, calcium, potassium, phosphorus, and whole blood sugar levels are determined in 18 calves with diarrhea and compared with the normal values. Some of these calves

were treated with a balanced electrolyte solution given intraperitoneally.

The findings indicate that in most cases a metabolic acidosis is present, varying from moderate to severe. Concomitant with the acidosis, variations are observed in the serum chloride, sodium, calcium, and potassium levels.

The significance of these findings is discussed. It is shown that by use of a balanced electrolyte solution many of the abnormalities can be corrected. In many instances, the early use of antibiotics and chemotherapeutic agents, by controlling the diarrhea, prevents electrolyte changes from becoming extreme. It is known that in diarrhea there may be changes in the intracellular as well as the extracellular electrolyte pattern and for this reason it is inadvisable to use solutions for parenteral injection containing only extracellular ions. Potassium in particular should be included in solutions used for this purpose.

The treatment of calf diarrhea with antibiotics and chemotherapeutic agents alone is apt to give disappointing results if the above mentioned changes are extensive. Variations from normal in acid-base balance and body electrolytes should be anticipated and treated before the changes become irreversible. Adverse effects were not noticed following the administration of the balanced solution but overhydration is a factor that must be considered. Further work involving either the fluid used in this study or other replacement solutions is indicated.

References

[1] Bland, J. H.: The Clinical Use of Fluid and Electrolyte. The W. B. Saunders Co., Philadelphia. 1952.

[2] Blaxter, K. L., and Wood, W. A.: Some Observations on the Biochemical and Physiological Events Associated with Diarrhea in Calves. Vet. Rec., *65*, (1953): 889-892.

[3] Cantarow, A., and Trumper, M.: Clinical Biochemistry. 4th ed. W. B. Saunders Co., Philadelphia. 1950.

[4] Darrow, D. C.: The Retention of Electrolyte During Recovery from Severe Dehydration Due to Diarrhea. J. Pediat., *28*, (1946): 515-540.

[5] Darrow, D. C.: Disturbances of Electrolyte Metabolism in Man and their Management. Bull. New York Acad. Med., *24*, (1947): 147-165.

[6] Darrow, D. C., Pratt, E. L., Flett, J., Gamble, A., and Wiese, H.: Disturbances of Water and Electrolyte in Infantile Diarrhea. Pediatrics, *3*, (1949): 129-156.

[7] Darrow, D. C., and Pratt, E. L.: Fluid Therapy, Relation to Tissue Composition and the Expenditure of Water and Electrolyte. J. Am. M. A., *143*, (1950): 365-375.

[8] Fox, C. L., Winfield, J. M., Slobody, L. B., Swindler, C. M., and Lattimer, J. K.: Electrolyte Solution Approximating Plasma Concentrations with Increased Potassium for Routine Fluid and Electrolyte Replacement. J. Am. M. A., *148*, (1952): 827-833.

[9] Gamble, J. L.: Deficits in Diarrhea. J. Pediat., *30*, (1947): 488-494.

[10] Hand, A. M., and Leininger, C. R.: Parenteral Fluid Therapy in Children. M. Clin. of North America, *34*, (1950): 53-70.

[11] Harrison, T. R.: Principles of Internal Medicine. The Blakiston Co., Philadelphia, (1951): 316.

[12] King, E. J.: Microanalysis in Medical Biochemistry. 2nd ed. J. A. Churchill Ltd., London, (1951): 23.

[13] McSherry, B. J., and Grinyer, I.: The pH Values, Carbon Dioxide Content, and the Levels of Sodium, Potassium, Calcium, Chloride, and Inorganic Phosphorus in the Blood Serum of Normal Cattle. Am. J. Vet. Res., *15*, (1954): 509-510.

[14] Mudge, G. H., Manning, J. A., and Gilman, A.: Sodium Acetate as a Source of Fixed Base. Proc. Soc. Exptl. Biol. and Med., *71*, (1949): 136-138.

Crystallization Patterns in Vaginal and Cervical Mucus Smears as Related to Bovine Ovarian Activity and Pregnancy

J. F. BONE, D.V.M., M.S.

Corvallis, Oregon

THE RELATIONSHIP between the secretions of the lower reproductive tract and the fertility status of cattle is not a new subject, but considerably more has been done with investigations of this phenomena in human beings. Tests using the crystallization patterns of the cervical mucus have been developed to a high degree in the human field but their application to animals is in its infancy. The cervical mucus crystallization phenomenon offers a fertile field for investigation, particularly in determining ovarian function and estrogen activity. Its value as a test for pregnancy in cattle, however, is open to question.

LITERATURE CITED

Cyclical changes in the cervical mucus of the cow have long been recognized. Williams [1] in 1917 made the observation that the cervical lumen becomes occluded by a sticky mucous plug during pregnancy, a condition markedly dissimilar to the normal mucous exudate at estrus. Woodman and Hammond [2] in 1925 described cyclic changes of the cervical mucus in cattle and stated that its liquefaction at estrus was a requisite to free passage of spermatozoa through the cervix. Hammond [3] in 1927 published observations upon cyclical changes in bovine cervical mucus.

Hammond [4] further stated that the consistency of the cervical mucus in cattle has a direct relationship to the functional activity of the corpus luteum, becoming thicker and more adhesive with an increase in lutein activity. Watson [5] in 1939 influenced mucous secretion by estrogen injections. Papanicolau [6,7] in 1945 and 1946 showed that when cervical mucus of women was spread on clean glass slides and allowed to dry, a fernlike crystallization took place. This was apparently most abundant at ovulation time. Pommerenke [8] in 1946 reported cyclical variations in human cervical mucus as regards amount, acellularity, viscosity, penetrability by sperm, and chemical content. Rydberg [9] in 1948 confirmed Papanicolau's observations and further reported that an analysis of the cervical secretion showed the crystallization to be due to the presence of chlorides expressed as NaCl in the mucin. He produced similar crystallization patterns by adding normal saline solution to egg albumin and saliva. Campos daPaz [10] in 1950 reported that the fernlike pattern can be used to determine whether or not the mucous secretion is favorable to the passage of normal spermatozoa. Absence of the crystallization pattern indicated an unfavorable environment, with consequent blocking or destruction of the sperm.

Roland [11] in 1952 published an extensive report dealing with use of the cervical mucous smear as a determinant for human estrogen activity, ovulation, and early pregnancy. Regarding early pregnancy he records a 99 per cent accuracy in diagnosing some 500 cases. Garm and Skjerven [12] in 1952 published studies of cervical mucous crystallization patterns in cattle, stating that the fernlike crystals appear during estrus, disappear during the lutein phase of the estrus cycle and can not be seen during pregnancy.

It was thought that further investigation of the crystallization properties of bovine cervical mucus might be of value, first to determine whether or not the lack of crystallization accurately reflected pregnancy, and second to determine whether or not there was a relationship between crystallization and known reproductive disorders. A third question, the relationship between the crystallization patterns of cervical and vaginal mucus was developed during the course of this study to determine what effect failure to collect an uncontaminated cervical mucus specimen would have upon the accuracy of the results.

MATERIALS AND METHODS

A total of 183 cattle of dairy and beef type were examined. Of these, 163 were animals presented for slaughter at a local abattoir. Mucous smears were obtained directly from the cervical os immediately after slaughter, and the carcass and reproductive organs were examined immediately thereafter. The remaining 20 cattle had known histories and were examined while living through the use of a special type of vaginal speculum and scraper shown in figure 1. The results were confirmed by checking the histories and by physical examination.

The mucus obtained from the slaughtered and living animals was spread thinly over the surface of clean glass microslides and allowed to dry. The slides were then examined microscopically under low power with reduced light. The presence of fernlike crystallization patterns, such as those

Approved for publication as technical paper 843 by the director of the Oregon Agricultural Experiment Station. Contribution of the Department of Veterinary Medicine, Oregon State College, Corvallis.

TABLE 1—Comparison of Test Results on Pregnant and Nonpregnant Cows

	Reaction				Reaction			
	No. pregnant	No. positive	No. negative	Accuracy (%)	No. nonpreg.	No. positive	No. negative	Accuracy (%)
Slaughtered	103	100	3	97.06	60	15	45	75.00
Living	10	10	0	100.00	10	4	6	60.00
Total	113	110	3	97.69	70	10	51	72.83

shown in figure 2, was classed as a negative reaction, while the absence of these patterns was considered positive for pregnancy (fig. 3).

It was observed that the fernlike pattern was found in the first, second, and fourth phases of the estrous cycle, or from a period of about three days pre-estrus to nine days postestrus, and that this pattern was definitely related to the estrogenic activities of the ovary. Progesterone activity, on the other hand, caused absence of these crystallization forms (table 2 and fig. 4). Care was taken to avoid contaminating the mucus specimens with halogens or halogen-containing compounds. A preliminary study, based on the observations of Rydberg,[9] revealed that halogen contamination of cervical mucus specimens was a distinct source of error.

It was found that any of the members of the halogen family in the form of their sodium and potassium salts would produce fernlike aborizations when added to neutral mucin, egg albumin, or positive cervical mucus. Further investigation revealed that human perspiration could also produce a negative reaction. Chlorine and iodine containing disinfectants gave similar results. However, blood chlorides as present in normal serum or tissue fluids failed to influence crystallization.

Instruments contaminated with halogen-containing compounds occasionally affected crystallization patterns, but it was found that washing in neutral soap and rinsing in distilled water prior to sterilization was sufficient decontamination to avoid false negative results in the cervical mucus specimens.

RESULTS

Specimens from the 163 slaughtered cows revealed that of the 115 which were positive to the test, 15 were open while the remaining 100 were pregnant. Of the 48 animals which were negative to the test, 3 were pregnant (table 1). Thus, without applying corrective factors, the test was 87 per cent accurate in determining pregnancy, with 15 false positives and 3 false negatives. No definite reasons can be given why the 3 pregnant animals showed a nonpregnant reaction. Possibly the slides may have inadvertently become contaminated with a halogen, or perhaps vaginal rather than cervical mucus was used.

The 15 false positives, however, can be accounted for. Postmortem examination revealed that all of these animals possessed well-developed and apparently functional corpora lutea. Ten of these 15 animals had adequate histories, which revealed that 6 had a history of anestrus and 4 had been in heat ten to fifteen days prior to slaughter.

Of the 6 animals with a history of anes-

TABLE 2—The Effect of Phase* of Estrus and Pathological Conditions upon Test Results in Nonpregnant Animals**

Phase	Normal	Pathological	Total	Positive	Negative	Per cent
			SLAUGHTERED			
1	3	10	13	0	13	100.0
2	11	1	12	0	12	100.0
3	5	10	15	15	0	0.0
4	3	17	20	0	20	100.0
Total	22	38	60	15	45	75.0
			LIVING			
1	3	0	3	0	3	100.0
2	2	0	2	0	2	100.0
3	4	0	4	4	0	0.0
4	1	0	1	0	1	100.0
Total	10		10	4	6	60.0
Combined total	32	38	70	19	51	72.83

* An arbitrary division of the estrous cycle according to ovarian activity, *see* figure 4.
Phase 1—Onset of heat to ovulation, 3 days.
Phase 2—Developing corpus luteum, 5 days.
Phase 3—Height of corpus luteum activity, 9 days.
Phase 4—Involution of corpus luteum, 4 days
** Includes all lesions and abnormalities.

trus, the ovaries of 4 contained large, loosely attached corpora lutea, having the typical yellow color, thickened capsule, reduced vascularization and degenerate cell-

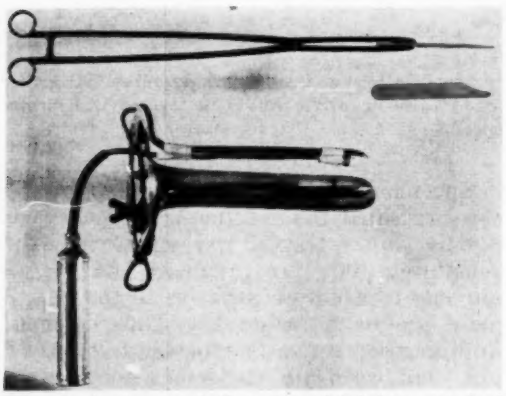


Fig. 1—Instrumentation for obtaining cervical mucus in living animals. (Top to bottom) Knowles forceps with scraper attached; scraper; equine vaginal speculum with lamp attached.

ular structure characteristic of persistent corpora lutea. The remaining 2 animals possessed cystic corpora lutea.

The 4 animals with a history of recent heat were obviously in the height of the lutein phase (phase 3) of estrus. The corpora lutea were well formed and intimately attached to the underlying ovarian stroma and had the characteristic yellow-brown color and rich vascularization of young structures.

Of the 5 animals without history, 2 showed old cystic corpora lutea. The remaining 3, judging from postmortem appearance, were in the height of the lutein phase of the estrous cycle.

If these 15 cases involving persistent corpora lutea and phase 3 animals are discounted, the test as applied to pregnancy is remarkably (97.06 %) accurate (table 1).

The tests on living animals showed an even higher correlation when those in the height of the lutein phase were eliminated. In the living animals, those which were pregnant reacted positively to the test, and in addition 4 which were not pregnant also reacted positively (table 1). These 4 had been in heat nine to eighteen days previously and were obviously in the height of the lutein phase. Examination of these animals shortly after the following estrus showed fernlike arborizations of the cervical mucus.

One of the most outstanding results was the early age at which pregnancy could be determined (table 4). Eleven of 12 slaughtered animals pregnant less than one month showed positive reactions. The ages of the fetuses were estimated by measurement of crown-rump length (table 3). Three embryos of 10 days of age or less were recovered from animals showing a positive reaction.

TABLE 3—Age* of Bovine Fetus

Months	Cm.	Months	Cm.
1	1.5	6	48.0
2	8.0	7	63.0
3	15.0	8	80.0
4	24.0	9	90.0
5	35.0	10	100.0

* Based on crown-rump length in centimeters.

The pregnant living animals were checked by rectal palpation. Two of these were so early in pregnancy that they could not be verified by palpation until some thirty days later. The remaining 8 were palpably pregnant at the time the mucous smears were obtained.

Further examination of the slaughtered cows revealed a high incidence of pathological conditions. This was not surprising since many probably had been culled for deficiencies of one sort or another. While interesting, these conditions apparently had no effect upon the results of the test unless they affected the ovaries.

Two sets of conditions encountered are worthy of remark. Three cases of cystic follicles showed negative reactions which was to be expected. However, 13 cases of cystic corpora lutea, 4 of which were associated with a history of anestrus also showed negative. Apparently, the proges-

TABLE 4—Results of Test Used on Cows at Various Stages of Pregnancy

Months pregnant	No. of cows	Positive reaction	Negative reaction	Per cent
Less than 1 month	12	11	1	92.0
1-2 months	21	21	0	100.0
2-3 months	10	9	1	90.0
3-4 months	15	14	1	92.6
4-5 months	19	19	0	100.0
5-6 months	9	9	0	100.0
6-7 months	10	10	0	100.0
7-8 months	3	3	0	100.0
8-9 months	4	4	0	100.0
Total	103	100	3	97.06

Fig. 2—Crystallization forms found in negative mucus indicating nonpregnancy.

terone activity of these structures was sufficient to suppress follicle development in these animals but insufficient to give a false positive reaction in the cervical mucus.

Of interest was the lack of correlation between vaginal and cervical mucus specimens. Despite the fact that vaginal mucus was obtained from areas immediately surrounding the cervix, the vaginal secretions gave a much higher incidence of false reactions and was a wholly unreliable medium upon which to base any conclusions regarding either pregnancy or estrogen activity.

Discussion

This study indicates that the cervical mucous secretions could serve not only as an indication of pregnancy but also as a possible method of determining estrogen activity. Vaginal mucous smears, however, are apparently less accurate.

At the present time, the use of cervical mucous smears is not recommended to supplant accepted methods of determining pregnancy and reproductive activity. Considerable sterile equipment is needed when more than 1 animal is to be tested and care must be taken to prevent contamination of either the mucus or the equipment with chlorides or other members of the halogen family in order to avoid false negative results. In the event that disinfectants are used it is imperative that they be tested to determine what effect they may have upon crystallization patterns. Also, one must be careful to obtain mucus only from the cervix, as contamination with vaginal mucus can influence results detrimentally.

The value of cervical mucus as a pregnancy test in cattle is enhanced by the keeping of accurate records of the animals in question. If histories are unknown, the test is only 87 per cent accurate, a percentage too low for field use. However, with good records, tests could be made at appropriate times during the estrous cycle and the false positives resulting from phase 3 animals reduced or eliminated. The remaining possibilities of error, e.g., persistent corpora lutea and an occasional false negative, would still remain. Al-

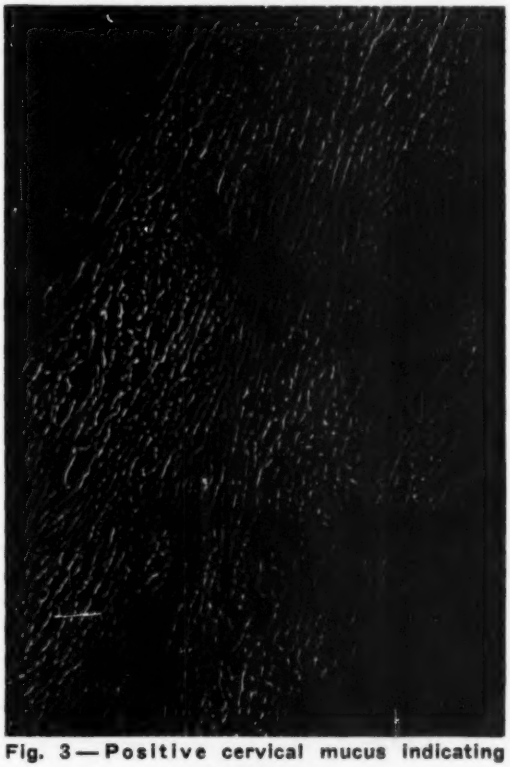


Fig. 3—Positive cervical mucus indicating pregnancy.

though adequate histories might reveal some of these cases, rectal examination would still be necessary to determine whether or not the animals in question were actually pregnant.

A proper test for determining pregnancy in cattle should not be based upon progesterone activity as the errors resulting from pathological conditions and from the lutein phase of normal estrus are sufficient to make its use impractical. Actually, a better test would be one that would accurately determine which animals are not pregnant. For the purpose, the cervical mucus test is not satisfactory, having an accuracy of only 72.83 per cent (table 1). As a test for estrogen activity, cervical mucous smears offer some interesting possibilities. The absence of the fernlike crystallization patterns during phase 3 pregnancy, and in conditions of anestrus due to persistent corpora lutea, indicates that the absence of crystallization can be used as a measure of progesterone activity of the ovary and, conversely, the presence of crystallization can be used as a measure of estrogen activity. A series of tests made over a complete estrous cycle would indicate the fertility status and the estrogen activity of the animal. It is quite possible that the employment of this test might have some value in determining the status of so-called functional infertility cases.

The cervical mucous test, while interesting and presenting a number of future possibilities, is still in an investigational stage. Considerably more work needs to be done before it can be recommended for routine clinical use.

CONCLUSIONS AND SUMMARY

1) Cervical mucus apparently has a definite relationship to ovarian activity and pregnancy in the normal bovine female. The crystallization patterns formed by allowing smears of cervical mucus to dry on clean glass slides have an inverse relationship to the progesterone activity of the gonads. During phases 1, 2, and 4 of the estrous cycle, the cervical mucus shows definite crystallization patterns which disappear during phase 3 and during pregnancy.

2) Vaginal mucus shows no such definite correlation and is an undependable medium for use in determining either pregnancy or estrogen activity.

3) Providing that accurate records are kept on the subject animals, cervical mucous smears can give an indication of pregnancy as early as ten days after conception. However, since the phenomenon of crystal-

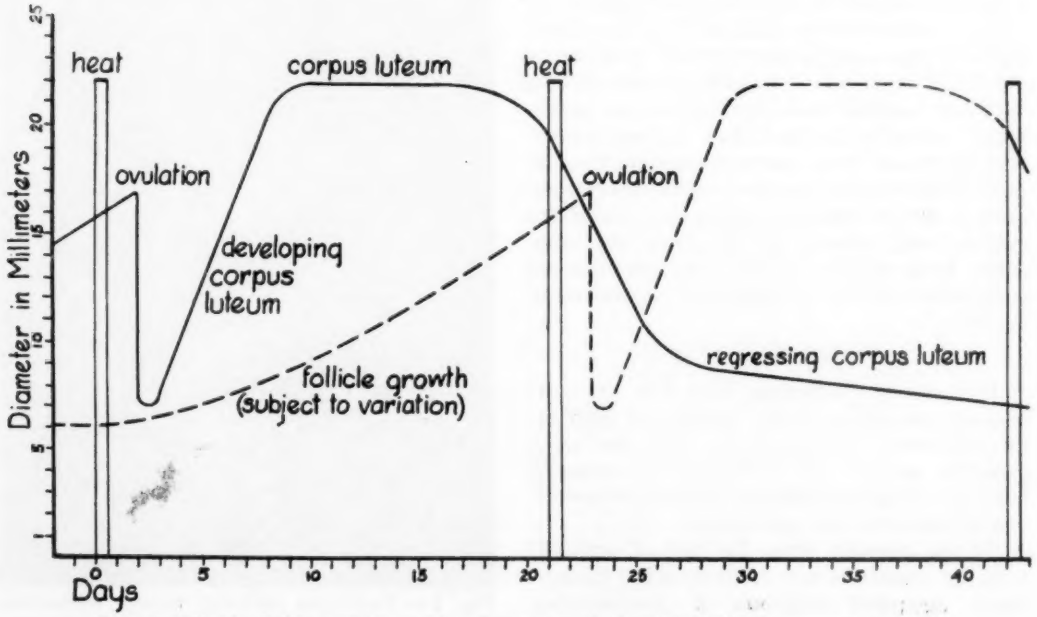


Fig. 4—Chart of the ovarian cycle of the cow (adapted from Hammond [3] p. 45).

lization is related to progesterone activity rather than to the actual presence or absence of an embryo or fetus, this test should not be used to replace present methods of pregnancy determination.

References

[1] Williams, W. L.: Veterinary Obstetrics. 4th ed. W. L. Williams, Ithaca, N. Y., (1947): 114.

[2] Woodman, H. E., and Hammond, J.: The Mucus Secretion of the Cervix of the Cow. J. Agric. Sci., *15*, (1925): 107-124.

[3] Hammond, J.: The Physiology of Reproduction in the Cow. Cambridge Univ. Press, London, (1927): 53-54.

[4] *Ibid.*: 94-95.

[5] Watson, M. C.: Effect of Cervical Secretions on Vitality of Spermatozoa. Canad. M. A. J., *40*, (1939): 542-543.

[6] Papanicolau, G. N.: Some Characteristic Changes in the Consistency of the Uterine Secretion. Anat. Rec., *91*, (1945): 293.

[7] Papanicolau, G. N.: A General View of the Vaginal Smear and its Use in Research and Diagnosis. Am. J. Obst. and Gynec., *51*, (1946): 316-327.

[8] Pommerenke, W. T.: Cyclic Changes in the Physical and Chemical Properties of Cervical Mucus. Am. J. Obst. and Gynec., *52*, (1946): 1,023-1,028.

[9] Rydberg, Erik: Observations on the Crystallization of Cervical Mucus. Acta obst. and gynec. Scandinav., *28*, (1948): 172-187.

[10] Campos da Pas, A.: Studies on Crystallization of Cervical Mucus and Its Relationship to Cervical Receptivity of Spermatozoa. Am. J. Obst. & Gynec. (suppl.), *61A*, (1951): 790-800.

[11] Roland, M.: A Simple Test for the Determination of Ovulation, Estrogen Activity, and Early Pregnancy Using the Cervical Mucous Secretion. Am. J. Obst. and Gynec., *63*, (1952): 81-89.

[12] Garm, O., and Skjerven, O.: Studies on Cervical Mucous for Early Diagnosis of Pregnancy and Endocrine Changes in the Reproductive Cycle in Domestic Animals. Nord. Vet-med., *4*, (1952): 1,098-1,103.

The Survival of Pathogenic Bacteria in Bovine Semen Preserved by Freezing

J. W. MACPHERSON, B.V.Sc., and N. A. FISH, D.V.M., D.V.P.H.

Guelph, Ontario

The present study was initiated with a view to furthering our knowledge of the effect of commonly used holding conditions, including freezing, on pathogenic bacteria in bull semen.

Literature Cited

Albertsen[1] reported on the bactericidal effect of antibiotics and sulfanilamide on strains of *Corynebacterium pyogenes, Brucella abortus,* and *Vibrio fetus* when added to egg-yolk semen diluent. Recovery of viable organisms from the diluent is reported after a storage period of twenty-four hours at temperatures varying from 5 C. to 30 C.

A satisfactory method of preserving bovine semen by freezing was described by Polge and Rowson[2] in 1952. Low temperature methods of preserving bacteria have been employed for many years.[3] However, the methods used differ from the "slow freezing" procedure presently utilized in preserving semen samples.

Materials and Methods

All the semen used in this study was collected at intervals from one bull by means of an artificial vagina. The semen was examined bacteriologically and no pathogenic organisms could be demonstrated.

Four species of bacteria, *viz., Listeria monocytogenes, Br. abortus, C. pyogenes,* and *V. fetus* were chosen for this experiment. The strains of *L. monocytogenes, C. pyogenes,* and *V. fetus* were recovered from aborted fetuses from herds in which artificial insemination was practiced. The strain of *Br. abortus* was isolated from bovine semen obtained from a naturally infected bull.

Listeria monocytogenes and *Br. abortus* were grown on tryptose agar slopes at 37 C. for forty-eight hours. *Corynebacterium pyogenes* was cultivated on 5 per cent horse blood agar slopes at 37 C. for forty-eight hours. A culture of *V. fetus* was obtained by cultivating the Vibrio strain on thioglycollate medium at 37 C. for forty-eight hours. The growth of all four organisms was removed with a phosphate buffered saline solution, pH 6.8. Bacterial suspensions were standardized to the density of tube number 1 of McFarland's nephelometer. Prior to the inoculation of the semen samples, the bacterial suspensions were diluted tenfold with additional phosphate buffered saline solution.

From the Ontario Veterinary College, Guelph.

Thanks are expressed to Dr. D. A. Barnum for conducting the sensitivity tests and to Miss Barbara Bradbury for technical assistance.

The semen sample was divided into four equal portions. Each of the four bacterial suspensions was added to a portion (3.0 ml.) of semen in an 0.25-ml. amount.

One quarter of each portion of the semen sample was diluted one part to 40 parts of milk diluent containing penicillin, 1,000 units per milliliter, and streptomycin, 500 μg. per milliliter at 30 C. A second quarter of each portion was diluted to the same extent in egg yolk citrate, consisting of one part egg yolk and one part 2.9 per cent sodium citrate with both antibiotics in the same concentration as above. The third quarter-portion was diluted in milk diluent without antibiotics, and the fourth quarter-portion in egg yolk citrate also without antibiotics. Half of each of the four quarter-portions was glycerinated and one half of the glycerinated portion was frozen in hermetically sealed vials;[4] these vials were stored at −79 C. The remaining portions, glycerinated and unglycerinated, were stored at 5 C. in hermetically sealed vials. The vials were examined for the presence of the bacteria after three days' storage. A duplicate set of the vials held at −79 C. was examined after seven days' storage. Table 1 summarizes the conditions under which the semen samples were held. For convenience, the lots of vials are numbered in the table according to the method of handling.

The method used to cool the diluted samples to 5 C. was as follows. Tubes of diluted semen were placed in a water bath at 30 C. The water bath was then placed in a refrigerator set at 5 C. Slow cooling was thus effected over a period of approximately four hours.

After the holding periods, the semen samples were centrifuged and the sediment inoculated onto tryptose agar plates containing 10 per cent horse blood, and into tubes of thioglycollate medium containing 10 per cent horse blood serum. The inoculated mediums were then incubated for four days aerobically and under reduced oxygen tension (20 % CO_2).

The semen sample sediments were then washed with a buffered sterile saline solution in order to remove the antibiotics. The washing procedure consisted of suspending the sediments in sterile saline. The semen portion containing *V. fetus* and buffered sterile saline was centrifuged at 3,500 r.p.m. for one hour. It was found that if lower speeds of centrifugation were used with this organism, Vibrio cells remained in the supernatant fluid. Centrifugation at 2,500 r.p.m. for one hour was sufficient for the other three organisms. The supernatant was discarded and the

sediment resuspended in additional saline. This washing procedure was repeated. When washing was completed the sediment was cultured again as above.

Six guinea pigs were blood tested and their serums were shown to be negative for Brucella agglutinins. Three of these animals were inoculated subcutaneously with 1.0-ml. quantities of the diluted semen samples containing viable Brucella organisms plus penicillin and streptomycin. At the same time, the other 3 guinea pigs were inoculated subcutaneously with 1.0-ml. quantities of the diluted semen samples containing viable Brucella organisms without antibiotics. Six weeks later the serums of all 6 guinea pigs were retested for Brucella agglutinins; 2 guinea pigs from each group were killed and cultures made of various tissues.

The four bacterial species employed were tested by the standard serial tube dilution method for sensitivity to penicillin and streptomycin. The sensitive level was taken as the lowest concentration of the antibiotic which inhibited growth.

RESULTS

Organisms of the four species were recovered in the absence of penicillin and streptomycin regardless of holding temperatures, diluent used, or the presence or absence of glycerol. When the semen sample was washed with buffered saline solution to remove the residual antibiotics before culture, the organisms were recovered. However, viable bacteria could not be demonstrated when the residual antibiotics were not removed by washing before culture.

The results of the sensitivity tests based on bacteriostatic activity appear in table 2 and indicate that growth of all species used was inhibited by both penicillin and streptomycin in concentrations much lower than those used in the semen diluent.

The results of all the animal tests with the samples containing Brucella were negative. The guinea pigs did not show agglutinins in their serums, and Brucella organisms were not recovered by bacteriological culture from these animals. Inoculations of control animals using semen samples without antibiotics also yielded negative results. It is possible, however, that infection may not have been achieved because of the degree of dilution.

TABLE 2—Sensitivity of the Strains of Corynebacterium Pyogenes, Brucella Abortus, Listeria Monocytogenes, and Vibrio Fetus to Penicillin and Streptomycin in Milk Diluent

	Penicillin		Streptomycin	
Organism	Resistant to units (ml.)	Sensitive to units (ml.)	Resistant to μg. (ml.)	Sensitive to μg. (ml.)
C. pyogenes	10.0	12.5	12.5	25.0
Br. abortus	10.0	12.5	25.0	50.0
L. monocytogenes	75.0	100.0	25.0	50.0
V. fetus	0.1	0.3	0.3	0.6

SUMMARY

1) Suspensions of *Brucella abortus, Listeria monocytogenes, Corynebacterium pyogenes,* and *Vibrio fetus* were added to semen samples which were handled as follows: (*a*) held at 5 C. for three days with and without the addition of penicillin and

TABLE 1—Viability of Listeria Monocytogenes in Diluted Semen

					Viable Bacteria		
						Antibiotics added	
Lot (No.)	Diluent	Time (days)	Temp. (C.)	Glycerol added	No antibiotics	before washing	after washing
1	egg yolk	3	5	none		−	+
2	milk	3	5	none		−	+
3	egg yolk	3	5	+		−	+
4	milk	3	5	+		−	+
5	egg yolk	3	−79	+		−	+
6	milk	3	−79	+		−	+
7	egg yolk	3	−79	+	+		
8	milk	3	−79	+	+		
9	egg yolk	3	5	none	+		
10	milk	3	5	none	+		
11	egg yolk	3	5	+	+		
12	milk	3	5	+	+		
13	egg yolk	7	−79	+		−	+
14	milk	7	−79	+		−	+
15	egg yolk	7	−79	+	+		
16	milk	7	−79	+	+		

+ Present; − absent; comparable results were obtained with the other three organisms tested.

streptomycin; (*b*) held at —79 C. for three- and seven-day-periods with and without the addition of penicillin and streptomycin.

2) Organisms of all four species survived the holding periods at 5 C. and —79 C. both with and without the addition of penicillin and streptomycin. However, viable organisms were demonstrated in the tests containing antibiotics only after the residual penicillin and streptomycin were removed by washing.

References

[1] Albertsen, B. E.: The Bactericidal Effect of Antibiotics in Semen Diluents (Preliminary Report). Proc. Fifteenth Internat. Vet. Cong., Stockholm, *2*, (1953): 731-734.

[2] Polge, C., and Rowson, L. E. A.: Long Term Storage of Bull Semen Frozen at Very Low Temperatures (—79 C.). Second Internat. Cong. Physiol. and Path. of Animal Reproduction and Artificial Insemination, (1952): 90-98.

[3] Proom, H., and Hemmons, L. M.: The Drying and Preservation of Bacterial Cultures. J. Gen. Microbiol., *3*, (1949): 7-18.

[4] Macpherson, J. W., and Herderson, J. A.: Low Temperature Preservation of Bull Semen. Proc. Book, AVMA (1953): 413-416.

Actinomyces Bovis Isolated from Six Cases of Bovine Orchitis
A Preliminary Report

ALICE KIMBALL, B.S.; M. J. TWIEHAUS, D.V.M., M.S.;
E. R. FRANK, M.S., D.V.M.

Manhattan, Kansas

THE MATERIAL for this investigation was obtained from 6 cases of bovine orchitis originating from various areas over the State of Kansas and presented at the Kansas State College Veterinary Clinic for treatment. Only unopened and untreated cases were cultured. *Actinomyces bovis* of the "smooth type" was isolated from all cases examined. These strains were culturally, morphologically, and biochemically identical with 65 strains isolated from fistulous withers and poll evil of horses,[1-3] with strains 8,373 and 8,374 from the American Type Culture Collection, and with two strains isolated from cases of human actinomycosis.

COLLECTION OF MATERIAL

The testicles were removed surgically and brought to the laboratory for bacteriological examination. The testicle was seared with extremely hot spatulas and opened aseptically. Pieces of the edematous testicle were removed and placed in Petri dishes, then introduced with sterile spatulas into deep tubes of melted dextrose agar.

CULTURAL CHARACTERISTICS

Dextrose Agar Shake Cultures.—Growth was abundant below 1 to 2 cm. Colonies were soft with finlike outgrowths (fig. 1) and were white frequently assuming a pinkish brown color with age. Sparsely seeded colonies may attain a diameter of 4 to 8 mm. (fig. 1).

Sodium Thioglycolate Broth.—There was a luxuriant fluffy growth which usually extended from the bottom of the tube to within 1 to 2 in. of the top.

Blood Agar.—Hemolysis occurred in shake cultures containing a small percentage (0.25 %) of blood and on blood agar plates containing 1 per cent dextrose, incubated anaerobically.

STAINING AND MORPHOLOGY

Morphology.—Actinomyces bovis is pleomorphic, typically diptheroidal and branching forms are common. Individual cells may exhibit swollen ends or centers. Curved forms are common but frequently assume definite angles rather than curves, suggesting that they may result from two or more organisms being hinged together (fig. 2). Frequently these organisms appear as short to moderately long irregular rods (fig. 3), this form being interchangeable with the diphtheroid.

Staining.—Young cultures are typically gram positive though the absorption or the decolorization of violet dye is not uniform, sometimes resulting in a chainlike appearance of gram-positive cells. Gram-positive characteristics are lost with age so that old cultures may appear typically gram negative. They are nonacid fast.

BIOCHEMICAL FEATURES

In all biochemical tests, large numbers of vigorously growing organisms were employed as the inoculum.

Gelatin.—All strains liquefied gelatin, usually within five to nine days; occasionally a strain required twenty-five days for

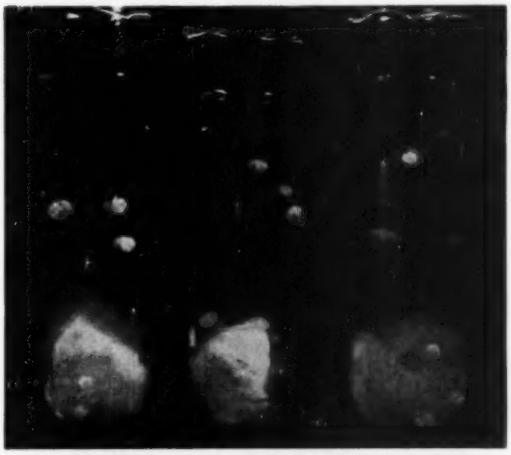


Fig. 1—Isolated colonies of Actinomyces bovis developing from direct inoculation with testicular material; incubation for eighteen days at 37 C.

Contribution 137, Department of Veterinary Medicine, Kansas Agricultural Experiment Station, Manhattan.

complete liquefaction. The medium contained 20 per cent gelatin (Difco), 2 per cent Bacto-tryptose, 0.5 per cent NaCl; no fermentable sugar was added.

Litmus Milk.—Every strain reduced litmus, and coagulated and digested the curd in litmus milk. Approximately half of the curd was digested within seven days; the remaining half digested more slowly. Bacto-litmus milk was found to be a satisfactory medium, if freshly prepared.

Indol.—Each isolate produced indol within two to five days in a medium containing the following materials: 2 per cent Bacto-tryptone, 0.5 per cent NaCl, 0.1 per cent sodium thioglycolate, 0.1 per cent agar, and no fermentable sugar. The Gore test was used to detect the presence of indol.

Ammonia.—All strains produced ammonia within two to four days in a broth composed of the following materials: 2 per cent Bacto-tryptose, 0.5 per cent NaCl, 0.1 per cent sodium thioglycolate, 0.1 per cent agar, and no fermentable sugar. The presence of ammonia was demonstrated by the "spot plate" method, 0.2 cc. of culture and 0.2 cc. of Nessler's reagent being used.

Hydrogen Sulfide.—All cultures produced H_2S within five to eight days, with the development of a more intense reaction during a longer incubation period. Bacto-lead acetate agar without sodium thiosulfate* was found to be a satisfactory medium. Melted tubes of agar were inoculated heavily by means of a Pasteur pipette just before solidification of the agar. The inoculum was expelled slowly as the pipette was withdrawn from the bottom of the tube.

* Difco Manual, 5th ed., Difco Laboratories, Inc., Detroit 1, Mich., (1935): 142.

Fig. 2—Diphtheroid form of Actinomyces bovis "S" type, as isolated from bovine orchitis.

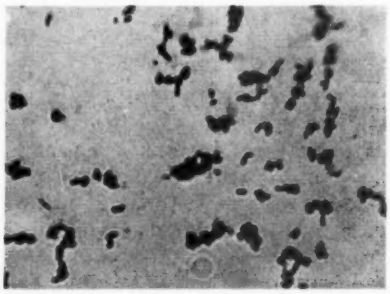


Fig. 3—Short-rod form of Actinomyces bovis "S" type, as frequently seen from initially isolated colonies.

Nitrate Reduction.—All strains reduced nitrate nitrogen to nitrite nitrogen within forty-eight hours in a medium containing the following materials: 2 per cent Bacto-tryptose, 0.5 per cent NaCl, 0.1 per cent sodium thioglycolate, 0.1 per cent agar, 0.5 per cent dextrose, and 0.1 per cent potassium nitrate.

Catalase Production.—All isolates produced an abundance of catalase within forty-eight hours in the same medium employed for nitrate reduction except that the potassium nitrate was left out.

Fermentation.—Fermentation tubes were prepared by adding a sterile solution of a neutral substrate to sugar-free broth which contained the following substances: 2 per cent Bacto-tryptose, 0.5 per cent NaCl, 0.1 per cent sodium thioglycolate, and 0.1 per cent agar, and adjusted to a pH of 7.1. The "spot plate" method was used to determine acid formation, since the addition of indicator to the medium proved to be inhibitory to growth of the organisms. Mannitol generally fermented more slowly than other substrates. No strain produced gas. All strains were identical in their fermentation reactions and the results were as follows: Acid formed in two to four days in mannose, dextrose, fructose, and glycerine; acid formed in two to seven days in galactose; acid formed in two to twenty-five days in mannitol and sorbitol; no acid was formed in maltose, lactose, sucrose, arabinose, xylose, dulcitol, raffinose, inulin, and salicin.

SUMMARY

The fact that *Actinomyces bovis* of the "S" type has been isolated from every case of bovine orchitis cultured in this laboratory suggests that this organism may be an important etiological factor in bovine orchitis. So far as the writers are aware, this is the first reported association of *A. bovis* with this disease.

References

[1] Kimball, A., and Frank, E. R.: The Isolation of *Actinomyces Bovis* from Fistulous Withers and Poll Evil. Am. J. Vet. Res., *6*, (1945): 39-44.

[2] Roderick, L. M., Kimball, A., McLeod, W. M., and Frank, E. R.: A Study of Equine Fistulous Withers and Poll Evil. Tech. Bull. 63, Kansas Agric. Exp. Sta., 1947.

[3] Roderick, L. M., Kimball, A., McLeod, W. M., and Frank, E. R.: A Study of Equine Fistulous Withers and Poll Evil. A Complex Infection with *Actinomyces Bovis* and Brucella. Am. J. Vet. Res., *9*, (1948): 5-9.

Use of Rabbit-Passaged Strains of the Nakamura LA Rinderpest Virus for Immunizing Korean Cattle

ROBERT C. REISINGER, D.V.M.; CHAI PONG MUN, D.V.M.; NAM SHIN LEE, D.V.M.

Pusan, Korea

SINCE 1945, the Republic of Korea has maintained an "immune zone" in the area immediately south of the thirty-eighth parallel for the purpose of preventing the spread of rinderpest from Manchuria which is known to be infected with rinderpest and from North Korea which is possibly infected.

The immune zone was established and maintained from 1945 through 1949 by immunizing all cattle in the zone by the simultaneous inoculation of the Nakamura III strain of lapinized rinderpest virus plus approximately 40 cc. of antirinderpest hyperimmune serum. The serum was necessary to counteract the virus, since it had been shown by Nakamura and Kuroda,[1] Nakamura,[2] and Lee[3] that approximately one third of Korean calves inoculated with lapinized virus alone died of rinderpest. In one experiment by the authors in August, 1952, of 6 calves inoculated with lapinized virus, 5 died of rinderpest, and the sixth recovered only after a protracted illness. According to Fukusho and his co-workers,[4-6] Nakamura,[7] and Reisinger,[8] Korean and Japanese cattle are relatively less resistant to rinderpest than is the Holstein-Friesian breed. We would therefore classify the Korean native cattle as a breed hyper-susceptible to rinderpest.

Due to the war no immunization program had been conducted in 1950, very few cattle had been immunized in 1951, and in the autumn of 1952 we had on hand enough serum to vaccinate only 15,000 cattle. At this time we received from Dr. Junji Nakamura* his strain of lapinized avianized (LA)[9] rinderpest virus. This virus had been developed from the lapinized virus by repeated passages in chicken embryos and was said to be so attenuated that it produced no adverse reaction in Japanese calves.

Laboratory experiments were then planned to determine (1) whether this virus could be safely and effectively used in Korean cattle as a vaccine; (2) how long it could be passaged in rabbits without increasing its pathogenicity for cattle; and (3) if rabbits might be employed as animals from which the vaccine could be produced. The second subject was especially important since the chicken embryo technique was impractical in our laboratory at that time. Also, production of vaccine from rabbits had been practiced with the Nakamura III lapinized virus in Korea, and this method was thought to have many advantages. Several lines of serial passages of the LA virus in rabbits have been established, and inoculation experiments with Korean calves have been conducted at different passage levels in each line. This work to date has yielded unique results in that the pathogenicity of the LA virus for calves has remained unchanged for many serial passages in rabbits, over 200 in one line. Furthermore, all the Korean calves inoculated have survived with little or no reaction and have acquired solid immunity against the bovine rinderpest virus.

In August 1952, a controlled field test was conducted with 181 cattle in two villages near the laboratory. After obtaining successful results in this trial, approximately 15,000 cattle in the area immediately south of the thirty-eighth parallel were vaccinated with the rabbit-passaged LA virus in November and December of 1952, and approximately the same number in 1953. This method of immunization has proved highly satisfactory in Korea.

Results of the laboratory experiments and the field vaccinations are briefly described below.

LABORATORY EXPERIMENTS

MATERIALS AND METHODS

Serial passages of LA virus in rabbits have been conducted in six lines. Starting virus, starting date, and number of passages in each line are shown in table 1. During passage, the virus was periodically tested for its pathogenicity and immunizing effect in calves, and also for its virus titer in calves and for rabbits. Materials and methods employed are as follows.

Calves.—The calves used for the experiments were Korean indigenous yellow calves 4 to 8 months in age and 120 to 260 lb. in weight. They were raised in the southern part of South Korea,

TABLE 1—Rabbit-Passage Lines Established

Starting LA virus	Date started	No. of Rabbit passages
AE224	2/12/52	228(continued)
AE185	6/ 4/52	20(discontin.)
AE226	8/ 6/52	81(continued)
AE261	8/30/52	9(discontin.)
AE237	10/ 4/52	10(discontin.)
AE264	8/17/53	10(discontin.)

From the United Nations Korean Reconstruction Agency (Reisinger), and the National Institute for Veterinary Research (Mun and Lee), Pusan, Korea.

* Director of the Nippon Institute for Biological Science, Tachikawa, Tokyo, Japan.

where rinderpest has not occurred for more than forty years, and where no vaccination program has ever been conducted, except for the field testing program of the authors in 1952.

Rabbits.—The rabbits used were a Japanese white breed of medium size, usually between 4 and 6 months of age, and weighing between 1,500 and 2,000 Gm.

Virus Source.—Orignal LA virus materials were wet or lyophilized chicken embryo spleen received from Tokyo. In four of the six rabbit-passage lines, the original materials, A-E224, A-E185, A-E226, and A-E261, respectively, were inoculated directly. In the remaining two lines, the original A-E226 had been passaged in our laboratory in chicken embryos by the same method as used by Nakamura and Miyamoto,[9] and the lines were started with the fresh chicken embryo spleen when they reached A-E237 and A-E264, respectively. The designation of the passage number of LA virus follows the nomenclature employed by Nakamura and Miyamoto; A-E185, for instance, represents:

A = 77 chicken embryo passages with 41 conjunctive rabbit passages;
E185 = 185 direct chicken embryo passages following the 'A' passages;
A-E185 is, accordingly, equivalent to 262 chicken embryo passages with 41 conjunctive rabbit passages.

Serial Passage in Rabbits.—For the starting LA virus, chicken embryo spleen, either wet or lyophilized, was suspended in physiological saline to 1:500, calculated from the weight of fresh tissue. After light centrifugation, 1 cc. of the supernatant fluid was intravenously inoculated into each of 2 rabbits. Three days later, blood was taken from 1 of the rabbits and directly transferred to the next group of 2 rabbits through the intravenous route at the dose of 0.5 cc. for each. In subsequent passages, the latter method was repeated.

Inoculation Experiments in Calves.—The mesenteric lymph node or the pool of lymph node, spleen, and blood was the material tested in calves. This was taken from the passage rabbit at the presumed peak of virus multiplication, *i.e.*, three days after inoculation. In the case of lymph node alone, the tissue was emulsified in 10 w/v of saline, filtered through gauze and then diluted in tenfold series as desired. With pooled tissues, whole mesenteric lymph nodes and spleens were emulsified in nine times w/v of defibrinated blood, filtered through gauze and saline added to make any desired dilution calculated from the weight of lymph node, spleen, and blood. The suspension was always prepared as quickly as possible after killing the rabbit, and inoculated subcutaneously to 1 or more calves at a dose of 2 or 5 cc. The calves were kept under clinical observation for two to three weeks, and then challenged by subcutaneous inoculation with 2 cc. of fresh virulent blood from a calf which had been inoculated with the laboratory bovine strain of rinderpest. Control calves were always simultaneously inoculated with the bovine challenge virus, and none has ever failed to die of the resultant typical acute infection. In some instances, several serial dilutions were inoculated to determine the minimum effective dose of the rabbit-passaged LA virus. In one experiment, challenge by the bovine virus was delayed for six and twelve months after LA vaccination to determine the duration of immunity conferred.

Virus Titration in Rabbits.—The virus content of tissues was titrated in rabbits as follows. Serial dilutions of the material were inoculated intravenously in rabbits, using 2 rabbits for each dilution and 1 cc. for each animal. The infection of each animal was judged by febrile reaction, and by the characteristic lesions of Peyer's patches in the intestine if the animal died or was killed within two weeks after inoculation, or by testing resistance to challenge with Nakamura III lapinized virus of those rabbits which survived the initial inoculation.

PATHOGENICITY AND INFECTIVITY IN RABBITS

The LA virus is less pathogenic to rabbits than the lapinized virus from which it originated. The thermal reaction and formation of necrotic lesions in Peyer's patches of the intestine are generally less prominent in rabbits infected with the LA virus than in those infected with the lapinized virus and are occasionally grossly imperceptible. According to Nakamura and Miyamoto,[9] the mortality rate among rabbits was less than 20 per cent with the LA virus while it was over 80 per cent with the lapinized virus. So far as these pathogenic features in rabbits are concerned, the LA virus regained its original lapinized character in a relatively small number of passages back into rabbits.

In the A-E224 line, such a reversion of the pathogenic character in rabbits appears to have occurred rather more slowly than the average. This line was, however, the richest in number of passage rabbits which were left alive for observation of their natural fate after inoculation and seems to be the most adequate example for the demonstration of the phenomenon. As seen in table 2, in this line, of the 18 rabbits which were used for the passage of LA virus up to the twentieth generation, and left for observation of natural termination, only 1 died. During the next ten passages, the mortality rate became 4 of 9 or 44 per cent, and since the thirtieth passage, the

mortality rate has exceeded 85 per cent which is comparable to that observed in rabbits infected with the original lapinized virus.

As for the infectivity of the virus to rabbits, virus titration of the infected rabbit lymph node or pooled tissues has been occasionally carried out in each rabbit passage line. Unfortunately, however, the tests were often incomplete, especially in earlier stages of the passages, and data obtained up to date are insufficient to give a definite conclusion as to the increase of infectivity during rabbit passage. However, the rabbit lymph node had a virus titer of 10^{-5} or 10^{-6} in earlier stages of the passages, and more frequently 10^{-6} and sometimes even 10^{-7} in the advanced stages.

TABLE 2—Mortality in Rabbits in AE224 Line

Rabbit passages	Mortality rate
1–10	1/10 (10 %)
11–20	0/8 (0 %)
21–30	4/9 (44 %)
31–40	8/9 (89 %)
41–50	6/7 (86 %)
51–60	8/9 (89 %)

According to Nakamura and Miyamoto,[9] chicken embryo spleens infected with the LA virus have a virus titer of 10^{-5} or 10^{-6} in rabbits.

PATHOGENICITY AND INFECTIVITY IN CALVES

As mentioned in the introduction, Nakamura III lapinized virus, though it has been remarkably attenuated for cattle, still maintains a strong pathogenicity for the hypersusceptible Korean cattle, and usually kills one third or more of inoculated calves. According to Nakamura and Miyamoto,[9] the LA virus has been further attenuated, and can be safely inoculated in Japanese calves which have a susceptibility to the lapinized virus comparable to that evidenced by Korean calves.

In one experiment conducted by the present authors, of 6 Korean calves inoculated subcutaneously with 2 cc. of a 1:100 suspension of lymph node of the 924th rabbit passage of the Nakamura III lapinized strain, all revealed clinical symptoms of rinderpest, and of them 5 died between seventeen and twenty-two days after inoculation. The temperature chart of one example, calf 107, is shown in table 4.

In two preliminary experiments, 4 Korean calves were inoculated with the original LA virus received from Tokyo, *i.e.*, 5 cc. of a 1:500 suspension of embryo spleen at the A-E224 and A-E226 passages. These animals revealed only slight thermal reaction and acquired solid immunity which completely protected them from any visible infection when subsequently challenged by the bovine rinderpest virus.

We had anticipated that with repeated rabbit serial passage the LA virus would regain its original pathogenicity for calves, but so far there has been no indication of this, and we suspect that the virus may be "fixed" as to its nonpathogenic character for calves. At least this is true through the latest rabbit serial passages tested in calves in each line, *i.e.*, the 220th passage in the A-E224 line, the eightieth in the A-E226 line, the twentieth in the A-E185 line, the ninth in the A-E261 line, the tenth in the A-E237 line, and the tenth in the A-E264 line. More than 120 calves have been inoculated in the laboratory with rabbit-passaged virus of relatively high concentration (2 or 5 cc. of 1:10 to 1:1,000 suspension of the rabbit lymph node, or pooled tissues consisting of lymph node and

TABLE 3—Reaction of Calves to Virus in AE224 Rabbit-Passage Line

No. of rabbit passage	Material inoculated	Tissue dilution	Duration of febrile reaction (days)								Total
			0	1	2	3	4	5	6	7	
4– 12	L	10^{-1}			1	2	1				4
16– 46	L	10^{-2}	1	2	2	2	2	1			10
56– 95	L	10^{-2}				2	2	1	1	1	7
105–135	LSB	10^{-2}	1		2	2		2			7
145–174	L	10^{-2}				5		1			6
180–204	L	10^{-2}	2	1	1						4
Total			4	3	6	13	5	5	1	1	38

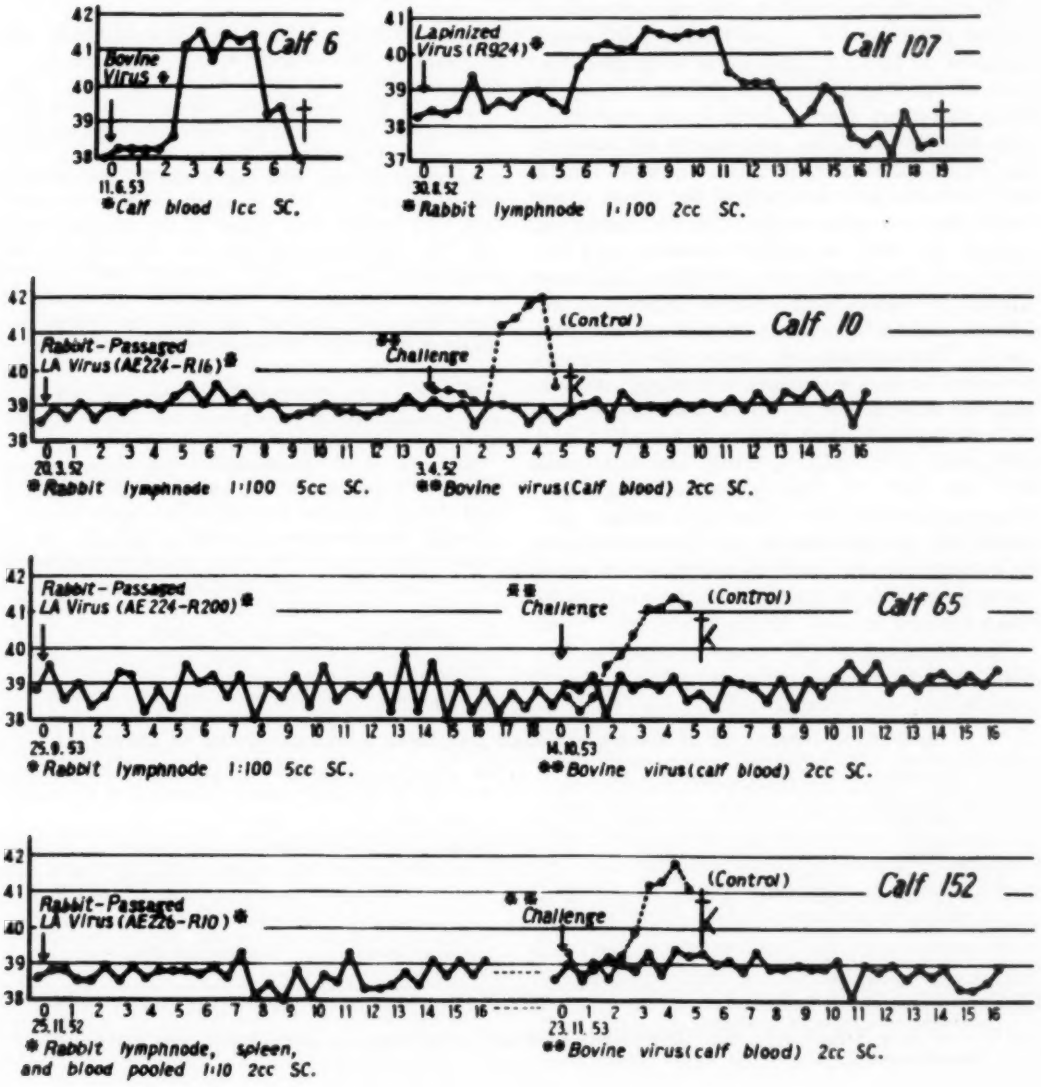
L—Lymph node, mesenteric; LBS—whole mesenteric lymph node and spleen, 1 part, plus defibrinated blood 9 parts.

All calves were solidly immune to challenge with the bovine rinderpest virus.

spleen 1 part and blood 9 parts), including 40 in the A-E224 line, 14 in the A-E226 line, 10 in the A-E185 line, 4 in the A-E261 line, 6 in the A-E237 line, and 7 in the A-E264 line. Without exception, these animals survived the inoculation with no adverse reaction and subsequently revealed complete resistance to the challenge bovine virus. The most common type of reaction in inoculated animals was a temperature rise usually after four to five days, lasting one to five days, and usually not exceeding 40.4 C. No other significant symptoms have been observed. In several animals, softening of feces was noticed, but it was difficult to judge whether this was due to the virus since this condition was not rare in our normal calves.

In table 3 are shown reactions in calves which were inoculated with the LA virus at various rabbit passages of the A-E224 line. No tendency toward increased pathogenicity of the virus for calves was observed as the passages advanced. Table 4 includes temperature charts of 2 calves (10 and 65) which were inoculated with

TABLE 4—Temperature Charts of Korean Calves After Inoculation with Bovine, Lapinized, and Rabbit-Passaged Lapinized-Avianized Strains of Rinderpest Virus



lymph node virus of the sixteenth and 200th rabbit passages in the A-E224 line.

In one experiment, 4 calves which had been inoculated with 2 cc. of a 1:10 pooled tissue (lymph node, spleen, blood) suspension of the tenth rabbit passage in the A-E226 line were challenged by the bovine virus, 2 of them six months and the remaining 2 twelve months after vaccination. The reaction to the LA inoculation was a slight temperature rise in 1 calf and almost none in the other 3. All these animals were found to be solidly immune to the bovine challenge virus. The temperature chart of calf 152 challenged twelve months after vaccination is shown in table 4.

The minimum calf-infecting or immunizing dose (m.i.d.) was titrated in seven samples of fresh lymph node or pooled tissues of rabbits at various passage levels in four different lines. Details of materials and results in calves are shown in table 5. In the A-E226 line, the test was made with only one sample. In each of the other three lines, the test was made with two samples, one at the fifth or earlier passage and the other at the tenth or twelfth. In these lines, the titer was always found to be higher at the later than at the earlier passage. Throughout all tests, the titer of samples from the earlier passages, excluding the one in the A-E185 line which was infective at 10^{-3} but not tested at 10^{-4} and 10^{-5}, was 10^{-4} or 10^{-5}, while the titer was 10^{-5} or 10^{-6} at the advanced passages. Among the samples from the earlier passages the pooled tissues of a rabbit which had been infected with the original chicken embryo virus were included, and their titer was 10^{-4}.

No remarkable difference in reaction has been noted between those calves inoculated with concentrated virus and those with highly diluted virus.

FIELD VACCINATION

MATERIALS AND METHODS

On Aug. 17, 1952, 180 Korean cattle were vaccinated with a suspension consisting of one part spleen and lymph node, nine parts of blood, and 90 parts of physiological saline. The dose was 2 cc. per animal administered subcutaneously. The virus used was the $AE_{226}R_8$ passage, and was harvested from rabbits eighty hours after intravenous injection. Immediately before vaccination, 151 prevaccinal serums were taken and 92 postvaccinal serums twenty to twenty-one days after vaccination. All serums were taken to the Nippon Institute for Biological Science for testing for the complement fixing (c.f.) antibody and for the virus neutralizing (v.n.) antibody. Full results of these tests are reported in detail by Nakamura, Kishi, Kiuchi, and Reisinger,[10] but a summary of the results is as follows:

TABLE 6—Results of Test for Complement-Fixing Antibody Content of Pre- and Post-vaccination Serums

	Prevaccination serums	Postvaccination serums
Positive	1 (0.7 %)	57 (62.6 %)
Doubtful	4 (2.6 %)	7 (7.7 %)
Negative	146 (96.7 %)	27 (29.7 %)
Total	151	91

Test for C.F. Antibody.—All the pre- and postvaccination serums were tested for their c.f. antibody content. Results are given in table 6.

Test for V.N. Antibody.—From extensive experience, it is known that animals can be considered to be immune to rinderpest when the c.f. antibody is demonstrated in the serum, but the reverse is not always true. The virus neutralization (v.n.) test (using embryonating chicken eggs and LA virus) is believed to be a more exact method for indicating immunity in cattle. All 27 c.f.-negative and 6 c.f.-doubtful postvaccinal serums were tested for their antibody content. As a con-

TABLE 5—Infectivity of Rabbit-Passaged Lapinized Avianized Virus to Calves

Virus Line		AE185		AE226		AE264		AE237
No. of rabbit passage		5	12	5	10	1	10	4
Tissue		L	L	LSB	LSB	LSB	LSB	LSB
Dilution of	10^{-1}	† ‡						† †
	10^{-2}		† †		† ‡	† ‡	†	
	10^{-3}	‡ ‡	‡ ‡	† ‡	† ‡	‡ ‡	† †	† ‡
	10^{-4}		† ‡	‡ ‡	† †	‡ ‡	†	
	10^{-5}		† —	— —	† ‡	— —	†	† —
	10^{-6}	— —	† ‡	— —	† ‡		—	

— No reaction and acquired no immunity; † No appreciable reaction, but acquired immunity; ‡ febrile reaction, and acquired immunity.

L—Lymph node, mesenteric; LSB—whole mesenteric lymph node and spleen, 1 part, plus defibrinated blood 9 parts.

trol, 10 c.f.-positive postvaccinal serums were also tested. Results are given in table 7.

As an additional test, vaccinated animal T52, whose postvaccinal serum was negative for c.f. antibody and positive for v.n. antibody in the above tests, was purchased and brought to the laboratory for a challenge test with bovine rinderpest

TABLE 7 — Results of Virus Neutralization (v.n.) Tests for Antibody Content of Pre- and Postvaccination Serums

	Prevaccination serums		Postvaccination serums	
c.f.	v.n. positive	v.n. negative	v.n. positive	v.n. negative
Positive	....	....	10	....
Doubtful	....	1	5	1
Negative	....	14	20	7
Total	0	15	35	8

c.f.—complement fixing.

virus thirty-eight days after vaccination. This animal was found to be solidly resistant to the bovine virus.

Also, 2 calves were vaccinated on the evening of Aug. 17, 1952, in the laboratory with the same batch of vaccine which had been used during the day for the field test, and both animals were entirely refractory to challenge with bovine rinderpest virus fourteen days later.

All vaccinated animals were carefully observed at appropriate intervals after vaccination and no untoward reactions were observed. All animals had been horn-branded and registered at the time of vaccination.

Following the successful completion of the preliminary field trial, approximately 15,000 cattle were vaccinated in the area just south of the thirty-eighth parallel during November and December of 1952, using the virus of the $AE_{224}R_5$ through $AE_{224}R_{40}$ passages, diluted and used as in the preliminary field trial.

In November and December of 1953, approximately 15,000 more cattle were vaccinated with the LA virus, using the A-E267 line, to maintain the immune belt below the thirty-eighth parallel. No adverse reactions to vaccination were reported following either of these two extensive projects. Since no rinderpest outbreaks have occurred in the Republic of Korea during these several years, no positive field evaluation of the efficacy of the vaccine has been proved. However, 100 prevaccinal and 48 postvaccinal serums were taken during the 1953 vaccination campaign and tested for c.f. antibody in a manner identical to that followed for the field test of 1952, except that the 1953 samples were collected fifteen and sixteen days after vaccination, instead of twenty and twenty-one days after. Full results of these tests are reported in detail in the paper of Nakamura, Kishi, Kiuchi, and Reisinger,[10] but a brief summary is given in table 8.

TABLE 8—Summary of Results of Tests of Pre- and Postvaccination Serums for Complement-Fixing (c.f.) Antibody Content Taken During 1953 Vaccination Campaign

c.f.	Prevaccination serums	Postvaccination serums
Positive	0 (0.0 %)	30 (64.5 %)
Doubtful	7 (7.0 %)	5 (8.7 %)
Negative	93 (93.0 %)	13 (27.0 %)
Total	100	48

By means of these two types of tests (c.f. and v.n.), antibody response was demonstrated in a high percentage of vaccinated animals. This seems to bear out results of laboratory experiments, in which all vaccinated animals were immune to challenge with the virulent bovine rinderpest virus.

SUMMARY AND CONCLUSIONS

1) Pathogenicity of the lapinized-avianized (LA) virus to calves is not appreciably influenced by numerous serial passages in rabbits. This has been proved for more than 220 passages in the A-E224 line, and 80 passages in the A-E226 line. These two lines are being continued.

2) Inoculation of the rabbit-passaged LA virus causes little or no thermal reaction in the hypersusceptible Korean calves, and confers solid immunity against the bovine rinderpest virus. This immunity was still complete six months and twelve months following vaccination in 2 calves challenged at each period.

3) After relatively few serial passages in rabbits, the LA virus regains pathogenicity for rabbits comparable to the pathogenicity of the original lapinized parent strain. In the A-E224 line, mortality rate among inoculated rabbits was 5.6 per cent during the first twenty serial passages, and over 85 per cent after the thirtieth passage.

4) More than 30,000 cattle were safely vaccinated with rabbit-passaged LA virus in the Republic of Korea during 1952 and 1953. No adverse reaction was observed in any vaccinated animal. Immune response, as indicated by production of complement fixation and virus neutralizing antibodies, occurred in more than 90 per cent of the vaccinated animals. All of more than 120 Korean calves vaccinated in the laboratory and subsequently challenged with bovine rinderpest virus were solidly immune.

5) The Nakamura LA strain of rinderpest virus furnishes a safe and efficacious means of immunizing Korean cattle without the simultaneous use of serum. It is believed that this strain will prove of value in immunizing other hypersusceptible breeds of cattle.

References

[1] Nakamura, J., and Kuroda, S.: Rinderpest: On the Virulence of the Attenuated Rabbit Virus for Cattle. Jap. J. Vet. Sci., *4*, (1942): 75-98 (Japanese original), 99-102 (English abstract).

[2] Nakamura, J.: Not published (data lost in Inst. of Vet. Res., Pusan, Korea).

[3] Lee, N.: Private communication.

[4] Fukusho, K., Furuya, K., and Ueno, K.: Rinderpest Infection in Japanese Cattle, as Compared in Holstein-Friesian Cattle. Oral Report at the Twenty-Fifth Meeting of the Jap. Soc. Vet. Sci., 1948.

[5] Fukusho, K., Ishii, S., and Takamoto, H.: Comparative Study on Infectiosity to Rinderpest of Japanese Black and Holstein-Friesian Cattle with Complement-Fixation Reaction. Rep. Govern. Exptl. Station Anim. Hyg., *26*, (1953): 35-39.

[6] Fukusho, K., Ishii, S., and Furuya, K.: Experiments on Susceptibility and Resistibility of Japanese Black and Holstein-Friesian Cattle against Lapinized Rinderpest Virus of Nakamura III Strain Serially Passaged in Formosa. Rep. Govern. Exptl. Station Anim. Hyg., *26*, (1953): 41-47.

[7] Nakamura, J.: Resistance of Japanese Indigenous Cattle to Rinderpest. A Report to the Second Asian Off. Internat. des Epizoot. Conf. at Bangkok, February, 1954.

[8] Reisinger, R.: Report to Public Health and Welfare Division, GHQ, Supreme Command Allied Powers, October, 1950.

[9] Nakamura, J., and Miyamoto, T.: Avianization of Lapinized Rinderpest Virus. Am. J. Vet. Res., *14*, (1953): 307-317.

[10] Nakamura, J., Kishi, S., Kiuchi, J., and Reisinger, R.: An Investigation of Antibody Response in Cattle Vaccinated with Rabbit-Passaged LA Rinderpest Virus in Korea. Am. J. Vet. Res. (To be published Jan., 1955).

A Study of Calcium in the Blood Serum of Egyptian Buffaloes Native and Crossbred Cattle

HUSSIEN REDA, M.V.Sc., and HASSAN SALEM, Ph.D.

Cairo, Egypt

CLINICAL analysis of blood samples is not as widely practiced in veterinary medicine as in human medicine.

LITERATURE CITED

In the early work of Newton,[8] it was shown that the average total serum calcium content of the blood of cows was 9.16 mg. of calcium per 100 cc. of serum. Godden and Duckworth [5] reported 10 to 12 mg. per 100 cc. for the total serum calcium in cattle. Jones [6] stated that the serum calcium tended to be high in tropical cattle and found it from 10.1 to 12.9 mg. per 100 cc. Payne, Clark, and Kingman [9] observed an increase in the serum calcium level in bulls over that found in cows. Their figures regarding age disagreed with those of Allcroft and Godden [1] showing higher calcium values in young calves than in their dams. Terri *et al.*[10] confirmed the results of Allcroft and Godden.[1] Newton [8] stated that the calcium percentage in serum was higher in pregnant than in nonpregnant cows, although Mullick and Pal [7] did not find any significant variation in the calcium during lactation and pregnancy. Allcroft and Godden [1] showed a slight decrease in the serum calcium of blood of cows immediately before and at parturition. Duckworth and Godden [4] showed that the total serum calcium attained its highest level a few days before calving. Blesser and Smith [2] stated that the calcium in the blood serum dropped 20 per cent in normally freshening cows at parturition. The calcium serum levels were 10.3 mg. of calcium per 100 cc. of serum five days prepartum, 8.7 mg. per 100 cc. at parturition, and 10.7 mg. per 100 cc. five days postpartum.

MATERIAL AND TECHNIQUE

The determination of total serum calcium was made on two herds belonging to the Faculty of Agriculture, consisting of 31 buffaloes and 24 cattle (14 Egyptian and 10 crossbred Shorthorn). The experimental animals were males, nonpregnant and pregnant females. The estimations of calcium on pregnant cases were made during the whole period of gestation.

Samples of blood were taken twice a week from all animals during the two months of the experiment in which no females were pregnant. The drawing of the samples was continued monthly on pregnant cases (10 buffaloes and 10 cattle) until delivery, then weekly after parturition for a month.

From the Faculty of Agriculture, Cairo University, Cairo, Egypt.

Blood samples were taken from the jugular vein in clean tubes. The method used was that of Clark and Collip.[3] The calcium level was calculated as milligrams of calcium per 100 cc. of serum.

RESULTS

Buffaloes.—As far as the authors know, there were no reports in the literature on the calcium content of the buffalo's blood. Normal buffaloes gave a calcium level between 8 to 12 mg. per 100 cc. with an average of 10 mg. per 100 cc. of serum.

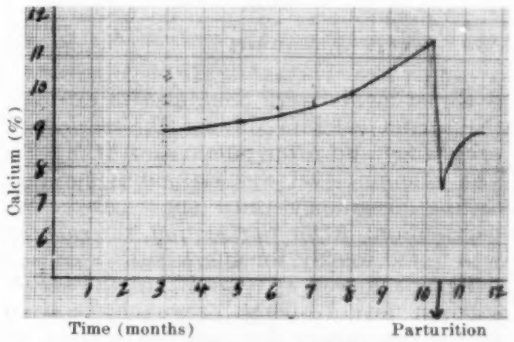


Fig. 1—Calcium level of buffaloes during pregnancy and after parturition.

Table 1 shows the averages of calcium level in the experiments on males, nonpregnant females, heifers, and calves.

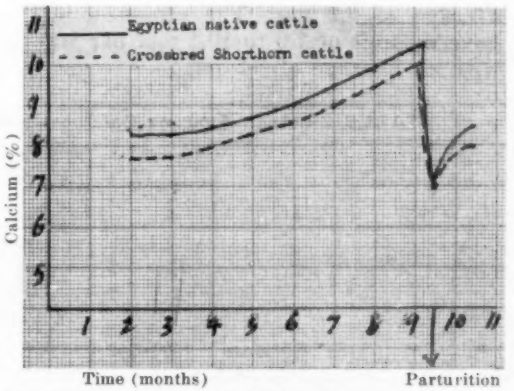


Fig. 2—Calcium level of Egyptian native cattle and of crossbred Shorthorn cattle.

TABLE 1—Mean Average Calcium Level in Buffaloes and Cattle

No. of Animals	Sex	Age	Calcium level (mg. per 100 cc. serum) (Max.)	(Min.)	(Ave.)
		BUFFALOES			
4	Bulls	4 to 8 years	11.7 ± 0.4	9.7 ± 0.7	11.2 ± 0.4
11	Cows	3 to 8 years	9.6 ± 0.4	8.2 ± 0.3	9.1 ± 0.5
9	Heifers	4 to 18 months	10.8 ± 0.8	9.0 ± 0.4	10.3 ± 0.3
7	Calves	3 to 6 months	10.2 ± 0.5	8.8 ± 0.6	9.8 ± 0.5
		EGYPTIAN CATTLE			
4	Bulls	6 to 10 years	10.7 ± 0.4	9.0 ± 0.4	10.2 ± 0.3
6	Cows	4 to 10 years	8.9 ± 0.5	7.7 ± 0.3	8.4 ± 0.4
2	Heifers	12 to 18 months	10.3 ± 0.3	8.5 ± 0.5	9.5 ± 0.1
2	Calves	6 months	10.2 ± 0.5	8.9 ± 0.4	9.5 ± 0.6
		CROSSBRED CATTLE			
3	Bulls	5 to 9 years	10.0 ± 0.1	8.2 ± 0.4	9.4 ± 0.5
4	Cows	4 to 7 years	8.6 ± 0.5	7.0 ± 0.2	8.0 ± 0.5
1	Heifers	12 months	10.0	7.8	8.5
2	Calves	6 months	9.8 ± 0.4	7.9 ± 0.1	8.9 ± 0.1

Experiments were done also to estimate the serum calcium during the period of pregnancy and after parturition (table 2). The calcium level was estimated monthly during pregnancy until one week before parturition. After parturition, the calcium was tested weekly for one month.

From figure 1, it is seen that the serum calcium level during pregnancy started rather low, about 9.0 mg. per 100 cc. of serum in the early pregnancy, and increased gradually, reaching the maximum of 11.5 mg. per 100 cc. about one week before delivery. After parturition, it decreased to 7.5 mg. per 100 cc., then gradually increased to normal.

Cattle.—The above experiments were repeated on Egyptian cattle and on crossbred Shorthorns for comparison. Normal Egyptian cattle gave a range of 7.3 to 11.0 mg. of calcium per 100 cc. of serum, with an average of 9.2 mg. The crossbred cattle gave a range of 7.0 to 10.1 mg. per 100 cc. with an average of 8.5 mg.

Table 1 indicates the averages of calcium level in experiments on males, nonpregnant females, and young cattle. From table 2, it appears that the calcium level in the crossbred animals was less than that in the Egyptian animals. The calcium level during pregnancy and after parturition was also estimated (table 2 and fig. 2).

The curves of the calcium level during pregnancy and after parturition in the Egyptian cattle, the crossbred cattle, and the buffaloes were parallel.

DISCUSSION

In general, the total calcium level in the serum of buffaloes was higher than in the cattle, either native or crossbred. The calcium level of normal Egyptian cattle is close to those of Newton.[5] The serum calcium level of the crossbred cattle was less than that of the Egyptian cattle. These results agreed with those of Jones.[6]

Diurnal and individual variations in the calcium levels of normal animals were no-

TABLE 2—Mean Average Calcium Level During Pregnancy and After Parturition

Calcium Level (mg. per 100 cc. of serum)													
Pregnancy (months)										Postpartum (weeks)			
2	3	4	5	6	7	8	9	10	part.	1	2	3	4
	BUFFALOES												
	9.1 ±.4	9.1 ±.3	9.3 ±.4	9.4 ±.5	9.7 ±.5	10.1 ±.4	10.7 ±.6	11.5 ±.5	7.6 ±.3	8.2 ±.4	8.8 ±.5	9.0 ±.8	8.8 ±.5
	EGYPTIAN CATTLE												
8.3 ±.7	8.3 ±.4	8.5 ±.8	8.7 ±.4	9.0 ±.5	9.5 ±.5	9.9 ±.2	10.4 ±.5		7.0 ±.8	7.5 ±.6	8.0 ±.7	8.0 ±.4	8.5 ±.6
	CROSSBRED CATTLE												
7.8 ±.3	7.8 ±.4	8.0 ±.8	8.3 ±.6	8.6 ±.6	8.9 ±.6	9.5 ±.3	9.9 ±.1		6.9 ±.5	7.4 ±.2	7.8 ±.5	7.9 ±.4	8.0 ±.4

ticed. One of the buffaloes gave about 6.4 mg. of calcium per 100 cc. of serum on the ninth month prepartum, which was about half the normal amount. Also, the same buffalo gave about 12.5 mg. of calcium per 100 cc. of serum when tested on the first week postpartum. This figure was very high compared with the normal levels. It is doubtful whether any significance should be attached to this variation since it was shown by only 1 buffalo.

The results in the buffaloes were similar to those of the Egyptian and crossbred cattle. Males had higher levels of calcium than females and age had no influence on the serum calcium levels. This agrees with Payne and Clark.[9] Pregnant cows or buffaloes gave a higher calcium level than the nonpregnant cows. When the calcium level was estimated during the period of gestation, it decreased gradually reaching its maximum in very late pregnancy, then fell below the normal level after parturition, and gradually rose again.

SUMMARY

1) Egyptian buffaloes had an average total calcium level of 10 mg. per 100 cc. of serum, which was higher than the Egyptian cattle with about 9.2 mg. per 100 cc. and the crossbred cattle with about 8.5 mg. of calcium per 100 cc. of serum.

2) Males had higher calcium levels than females, and pregnant animals had a higher level than nonpregnant animals.

3) The calcium level increased during the period of gestation, reaching a maximum level a week before parturition, falling below normal after parturition, then later rising to normal.

References

[1] Allcroft, W. M., and Godden, W.: Changes in Calcium and Magnesium of Serum and in the Inorganic Phosphorus of the Blood of Cows. Biochem. J., *28*, (1934): 1004.

[2] Blosser, T. H., and Smith, Vearl R. Blood Serum Levels of Citric Acid and Calcium in Normal Parturient Cows. J. Dai. Sci., *33*, (1950): 81.

[3] Clark, E. B., and Collip, J. B.: A Study of the Tisdall Method for the Determination of Blood Serum Calcium with Suggested Modifications. J. Biol. Chem., *63*, (1925): 461.

[4] Duckworth, J., and Godden, W.: The Partition of Serum Calcium About the Time of Parturition in the Dairy Cow. J. Dai. Sci., *6*, (1940): 9.

[5] Godden, W., and Duckworth, J.: A Long Term of Serum Calcium in Ayrshire Cows. J. Dai. Res., *6*, (1940): 15.

[6] Jones, E. R.: The Effect of Tropical Environments on the Blood and Milk of Cattle. Vet. Rec., *55*, (1943): 128.

[7] Mullick, D. N., and Pal, A. K.: Studies of Some Hematological and Chemical Constituents in the Blood of Normal Cattle. Indian J. Vet. Sci., *13*, (1944): 146.

[8] Newton, O. M.: Blood Calcium of Cattle. Rev. Med. Vet., Buenos Aires, *16*, (1934): 12.

[9] Payne, M. G., Clark, A. G., and Kingman, H. F.: Blood Levels of Calcium and Inorganic Phosphorus in Hereford Cattle. J. Agric. Res., *72*, (1946): 357.

[10] Terri, A. E., Keener, H. A., and Marrow, K. S.: Studies on the Chemical Composition of Calf Blood. J. Dai. Sci., *29*, (1946): 663.

Control of Pasture-Acquired Infections of *Nematodirus Spathiger* and *Haemonchus Contortus* in Lambs with Phenothiazine-Salt Mixture

J. H. TURNER, M.S., and M. L. COLGLAZIER, B.A.

Beltsville, Maryland

As a sequel to the elucidation of the pathogenicity of *Nematodirus spathiger*, a common trichostrongylid nematode of sheep, by Kates and Turner,[3] experiments were undertaken on the chemotherapeutic control of this species. A 1:9 phenothiazine-salt mixture, administered free-choice, was chosen as the test regimen largely because of the promising result of an earlier experiment [4] in which Nematodirus spp. were incidentally involved and partly, also, because of the wide reliance that is placed upon this regimen as a general parasite-control measure. Despite scattered reports of a variable efficacy against Nematodirus spp. of a few well-known drugs, namely, phenothiazine, carbon tetrachloride, tetrachlorethylene, hexachloroethane, and cupric drench, none of these, used therapeutically, is of uniform or established efficacy and the employment of one or another of them in this manner seemed, therefore, to offer little, if any, prospect of successful control. In Swales' report [5] on experiments somewhat similar to those reported herein, in which he tested the value of phenothiazine-salt mixture for the control of natural infections of several sheep nematodes, the results concerning the control of Nematodirus spp. were inconclusive, probably because of the small numbers of these worms present in his animals.

Although the experiment reported herein was originally designed to test the efficacy of a phenothiazine-salt mixture against pure *N. spathiger* infections in lambs on pasture, the experimental lambs unavoidably acquired infections of *Haemonchus contortus*, the large stomach worm of sheep. However, this afforded the opportunity to study the interaction of these species in lambs having access to (1) phenothiazine-salt mixture and (2) salt only. Certain results of these studies have been briefly reported in abstract form.[1,8] The studies were carried out during successive years, 1951 and 1952; therefore, the procedures employed and results obtained are discussed as the "first trial" (first year) and "second trial" (second year).

From the Animal Disease and Parasite Research Branch, Agricultural Research Service, U.S.D.A., Beltsville, Md.

The authors acknowledge the advice and assistance of Drs. K. C. Kates, F. D. Enzie, L. R. Sinclair, and A. O. Foster.

Experimental Procedures

First Trial.—For this experiment a ½-acre pasture naturally contaminated with larvae of *N. spathiger* was divided into two comparable ¼-acre pastures by a woven wire fence. There was also available a ¼-acre uncontaminated pasture, which had not been grazed by sheep for several seasons, comparable in other respects to two ¼-acre contaminated pastures. The forage on these pastures consisted mainly of Kentucky Blue grass and mixed wild grasses.

Early in May of the first year, 12 lambs of mixed breed, approximately 2 months old, were divided into three groups of 4 lambs each; the total weight of each group was approximately equal when placed on the pastures. These three groups were designated group A (treated, on contaminated pasture A), group B (untreated, on contaminated pasture B), and group C (untreated, on clean pasture C). The lambs of group A had access at all times to a 1:9 phenothiazine-salt mixture to which was added a small amount of grain, the mixture being placed in a readily accessible box. The other two groups were offered salt only in the same manner. The average consumption of phenothiazine per lamb for the duration of the experiment was 0.504 Gm., or 5.04 Gm. of the mixture, per day for group A. The lambs of group B consumed 4.59 Gm. of plain salt per lamb per day. Consumption of salt by group C lambs (4.32 Gm.) did not differ significantly from that of group B lambs.

The lambs were observed daily for clinical symptoms of parasitism and, on a weekly basis, hematocrit levels and body weight were determined and differential fecal egg counts made. All possible precautions were taken to prevent extraneous and cross contamination of the pastures during the course of the experiment.

On July 16, about midway in the course of the experiment, 2 lambs, with high egg counts, from each of groups A and B were killed in order to compare the parasite burdens of the lambs on these two pastures. This ended the first phase of

the experiment at eleven weeks. The lambs chosen for examination were those showing the highest counts of *H. contortus* eggs; all but 1 had higher *N. spathiger* egg counts than the lambs not destroyed. Thereafter, 2 lambs from pasture C were placed on each of pastures A and B for the remaining six weeks, the second phase of the trial. These lambs at the time of transfer had developed light *H. contortus* infections. The transfer of lambs from pasture C to pastures A and B was done to ascertain the rapidity and extent of acquisition of parasitic infections in lambs, exposed and unexposed to phenothiazine-salt mixture, during the latter part of the grazing season and, incidentally, to increase the number of principals and controls. The experiment was terminated late in August, the seventeenth week after lambs were first placed on the pastures, at which time the lambs were killed and their parasites recovered and counted.

Second Trial.—This was essentially a duplication of the first trial, except for certain necessary changes, but the duration was twenty-four weeks instead of seventeen. The same pastures were used. They were unoccupied from late August of the first year to early May of the second year, at which time the second trial began. However, the arrangement was changed in that the fence dividing pastures A and B was placed at right angles to its location the preceding year. This was done to secure an equivalent carry-over contamination of parasite larvae from the preceding year. It had been evident from the results of the first trial that fewer eggs were deposited by the lambs on the original pasture A than on B (fig. 1). Moreover, to replenish the contamination of *N. spathiger* on pastures A and B, about 100 lb. of droppings containing eggs of this parasite, obtained from experimentally infected lambs, were equally divided and distributed over the two pastures. Pasture C remained unchanged, except for the residual carry-over of larvae from the preceding year.

Early in May of the second year, 12 Shropshire lambs, approximately 2 months of age, were divided, as before, into three comparable weight groups of 4 lambs each. The same group and pasture designations were used, the lambs of group A having access to phenothiazine-salt mixture, while those of groups B and C were offered salt only. The average consumption of phenothiazine per lamb for the duration of this experiment was 0.282 Gm., or 2.82 Gm. of the mixture, per day for group A. The lambs of group B consumed 3.23 Gm. and those of group C, 3.27 Gm. of plain salt per lamb per day. Both the phenothiazine and the salt consumption in this trial was lower than in the first trial.

Additional data were obtained in this trial on the live carcass grades of the surviving lambs at the termination of the experiment.*

* Acknowledgment for these services is extended to Messrs. D. A. Spencer, C. G. Potts, and R. L. Clark of the Animal Husbandry Research Branch, Agricultural Research Service, U.S.D.A., Beltsville, Md.

Finally, because of drought and inadequate forage, supplemental feed consisting of 1½ lb. of alfalfa hay per lamb per day was offered to each group from July 31 to September 26. Late in October, after twenty-four weeks on pasture, the surviving lambs were killed and their parasites recovered and counted.

Laboratory Procedures. — The procedures employed were uniform during both trials. Blood was collected by jugular vein puncture, and the packed red cell volume in percentage (hematocrit) was determined by standard hematological methods.

Fecal specimens were obtained rectally and differential counts of *H. contortus* and *N. spathiger* eggs were done by the direct centrifugal flotation method, with saturated saline solution as the flotation medium, as described by Turner.[7] The abbreviation "e.p.g." used herein refers to eggs per gram of feces counted by this method.

When lambs died or were killed at the end of the experiments, all possible care was taken to recover all immature and mature worms from the gastrointestinal tract. The worms and ingesta obtained from the abomasum and small intestine of each lamb were placed in separate containers, physiological saline solution was added and, after sedimentation, some of the coarser particles of ingesta were removed by carefully decanting the supernatant fluid. Thereafter the material from each organ was stored in jars, to which dilute formalin was added as a preservative, until the worm counts were made. In doing the worm counts, water was added to the material in the jars to attain a standard volume of 2,000 ml. and, from this total volume, 2, 20-ml. aliquots were taken after thorough mixing. The helminths in these samples were identified and counted, and the

TABLE 1—Weight Gains or Losses of Medicated and Unmedicated Lambs on Contaminated Pastures During First and Second Trials

Trial	Weeks on pasture	No. of lambs	Period on pasture	Total weight (lb.) Group A (medicated)	Group B (unmed.)
First	0	4	May 5	117.5	117.0
			to		
	11	4	July 16	163.0	158.5
			Change	45.5	41.5
	0	2	May 5	59.5	66.5
			to		
	17	2	August 28	93.5	64.0*
			Change	34.0	−2.5
	0	2	July 17	107.0	99.5
			to		
	6	2	August 28	101.0	73.0
			Change	−6.0	−26.5
Second	0	4	May 8	109.0	109.5
			to		
	24	4	October 27	207.0	164.0**
			Change	98.0	54.5

* 1 lamb died August 20; terminal weight included; ** 1 lamb died September 30; terminal weight included.

total number present in the individual lambs were calculated by using the proper dilution factor. When the number of nematodes was too small to count with reasonable accuracy by this dilution method, direct counts were made of all the worms present.

RESULTS

Because the results obtained in these trials are presented rather fully in tables 1 and 2 and in figures 1 to 5, and to a considerable extent are self-explanatory, only general trends, main points of interest, and data not presented in the tables and figures will be discussed.

First Trial.—Once substantial infections were established in the lambs grazing the contaminated pastures, the animals on the phenothiazine-salt mixture (fig. 1 A, B, C) had lower fecal egg counts of *N. spathiger* and *H. contortus* throughout the experiment than the comparable lambs on salt only (fig. 1 A′, B′, C′). The early peak of *N. spathiger* egg counts in June and the much later peak of *H. contortus* egg counts in lambs of groups A and B showed that the initial contamination of the pastures was heavy with *N. spathiger* larvae but very light with *H. contortus*. The egg count data recorded for 2 lambs on contaminated pastures A and B for the first eleven weeks of this trial (fig. 1 A, A′) are essentially the same as those recorded in the graphs involving 4 lambs over this same period (fig. 1 B, B′). These 2 lambs, however, remained on the contaminated pastures for the full term of the experiment. The trend of egg counts for the medicated lambs, which were initially exposed to pasture A and remained thereon either for eleven or seventeen weeks, was that the *N. spathiger* egg counts reached an early peak and then declined to low levels in a few weeks, whereas the *H. contortus* egg counts were initially very low, rising to only moderate peaks in later weeks (fig. 1 A, B). The trend of the egg counts for the unmedicated lambs, comparable to those above, was initially the same but, in later weeks of the experiment, the *N. spathiger* egg counts rose to much higher peaks than in the medicated lambs (fig. 1 A′, B′). Although the *H. contortus* egg counts increased more rapidly in the 2 pairs of lambs placed on pastures A and B in midsummer (fig. 1 C, C′) than in the original lambs on these pastures, the egg count trend for these medicated and unmedicated lambs was similar to that for the others on the same pastures. From the egg count data alone, therefore, the control value of the medication was clearly demonstrated for both parasite species. Lamb 214 from pasture B died during the sixteenth week of this trial. The highest fecal egg counts (fig. 2) and the greatest number of *N. spathiger* (fig. 3A) were obtained from this animal.

Weight changes (table 1) of the lambs employed in this trial showed group A lambs gained 4 lb. more than group B lambs by July 16. The weight differential between the medicated and unmedicated pair of lambs remaining on the pastures seventeen weeks was 36.5 lb. Although both pairs of lambs transferred to pastures A and B on July 17 lost weight, the terminal total weight loss of the unmedicated lambs was 20.5 lb. greater than for the medicated lambs. All lambs of pasture A on medication maintained better weights than unmedicated lambs on pasture B. Group C lambs, however, on the clean pasture and without the benefit of medication gained 44.0 lb. more than group A lambs during the first eleven weeks on pasture.

Diarrhea occurred in the unmedicated lambs on pasture B during the period indicated (fig. 1 A′, C′; 3A), whereas there was no diarrhea in the medicated lambs.

The red blood cell level changed little in the medicated lambs on pasture A during the first eleven weeks, average hematocrit

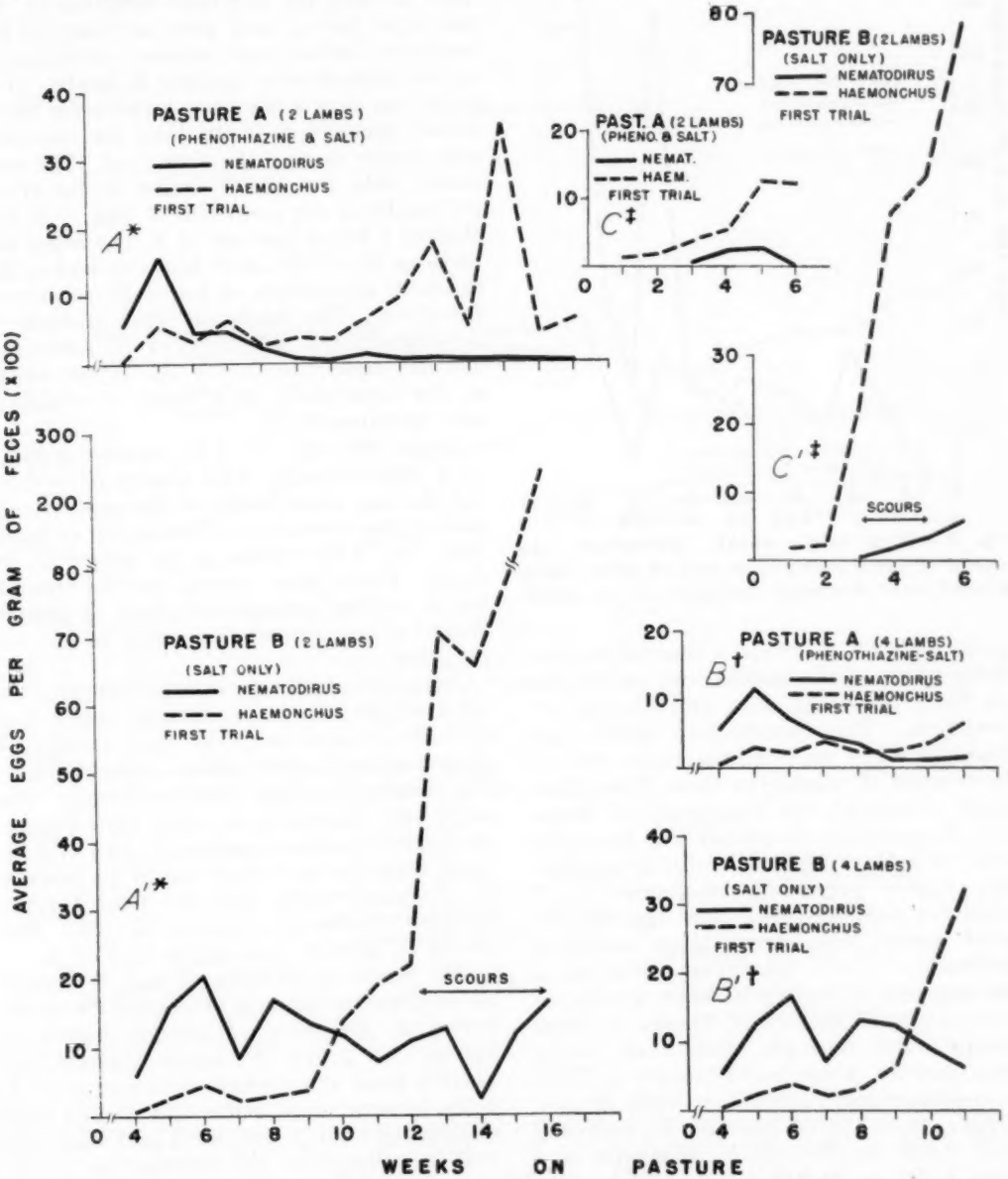
TABLE 2—Parasites Recovered* at Autopsy of All Lambs Exposed to Contaminated Pastures A and B During First and Second Trials

Trial	Parasites	Pasture A (medicated)	Pasture B (unmedicated)	Worm-count-ratio (pasture A to B)
First**	*Nematodirus spathiger*	18,130 (44 %)	518,700 (43 %)	1:28.0
	Haemonchus contortus	16,809 (47 %)	55,495 (71 %)	1: 3.3
Second†	*Nematodirus spathiger*	28,300 (50 %)	101,600 (33 %)	1: 3.6
	Haemonchus contortus	6,300 (33 %)	190,200 (90 %)	1:30.0

* Total worm counts include both immature and mature worms; immature worms are given as percentage of total.

** 6 lambs on each pasture; † 4 lambs on each pasture.

levels decreasing only slightly from 35.5 to 31.0 per cent, whereas the average hematocrit level decreased significantly in the unmedicated lambs on pasture B over the same period, falling from 34.7 to 25.5 per cent. During the last six weeks, the average hematocrit level of lambs on pasture A dropped from 31.2 to 26.2 per cent, whereas that of lambs on pasture B fell from 29.9 to 9 per cent. The hematocrit level was estimated to be less than 8 per cent in the lamb (214) that died. Thus, a slight ane-



* A, A', lambs exposed entire experimental period; † B, B', lambs exposed during first eleven weeks, including those of A and A'; ‡ C, C', lambs transferred to contaminated pasture and exposed during last six weeks.

One lamb died August 20, sixteen weeks after being placed on pasture.

Fig. 1—First trial, average weekly differential egg counts of lambs on contaminated pastures.

mia developed in the medicated lambs and an acute anemia in the unmedicated lambs.

The postmortem worm counts obtained from the medicated and unmedicated

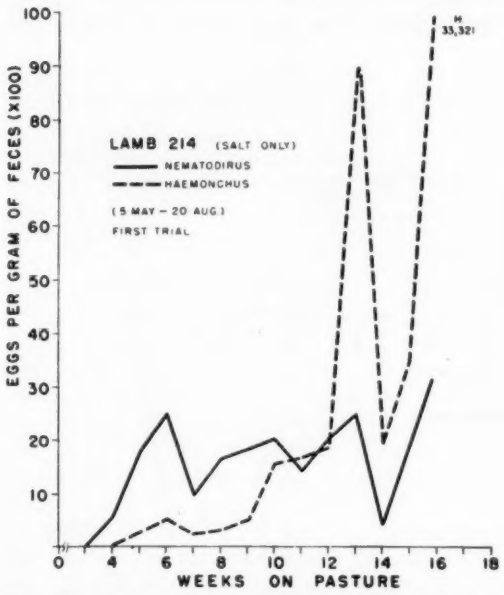


Fig. 2 — First trial, weekly differential egg counts of lamb dying near end of trial; lamb scoured over five-week period prior to death.

lambs (table 2, first trial), showed the beneficial effect of the medication, as did also the fecal egg-count data and clinical observations. The unmedicated lambs harbored 28 times more *N. spathiger*, and 3.3 times more *H. contortus* than those medicated. Although the percentage of immature *N. spathiger* recovered was about the same in both groups of lambs, a considerably higher percentage of immature *H. contortus* was recovered from the unmedicated lambs than from those receiving medication. There was great variation in the numbers of worms of both species recovered from individual lambs of both groups. For example, individual lambs from pasture A harbored from 0 to 7,700 *N. spathiger* and from 280 to 6,080 *H. contortus;* those from pasture B harbored from 2,600 to 260,000 *N. spathiger* and from 4,260 to 24,500 *H. contortus.* The very small numbers of *Strongyloides papillosus,* Ostertagia spp., *Trichuris ovis,* and *Moniezia expansa* recovered from the lambs are not recorded, as they were not considered of any significance in this experiment.

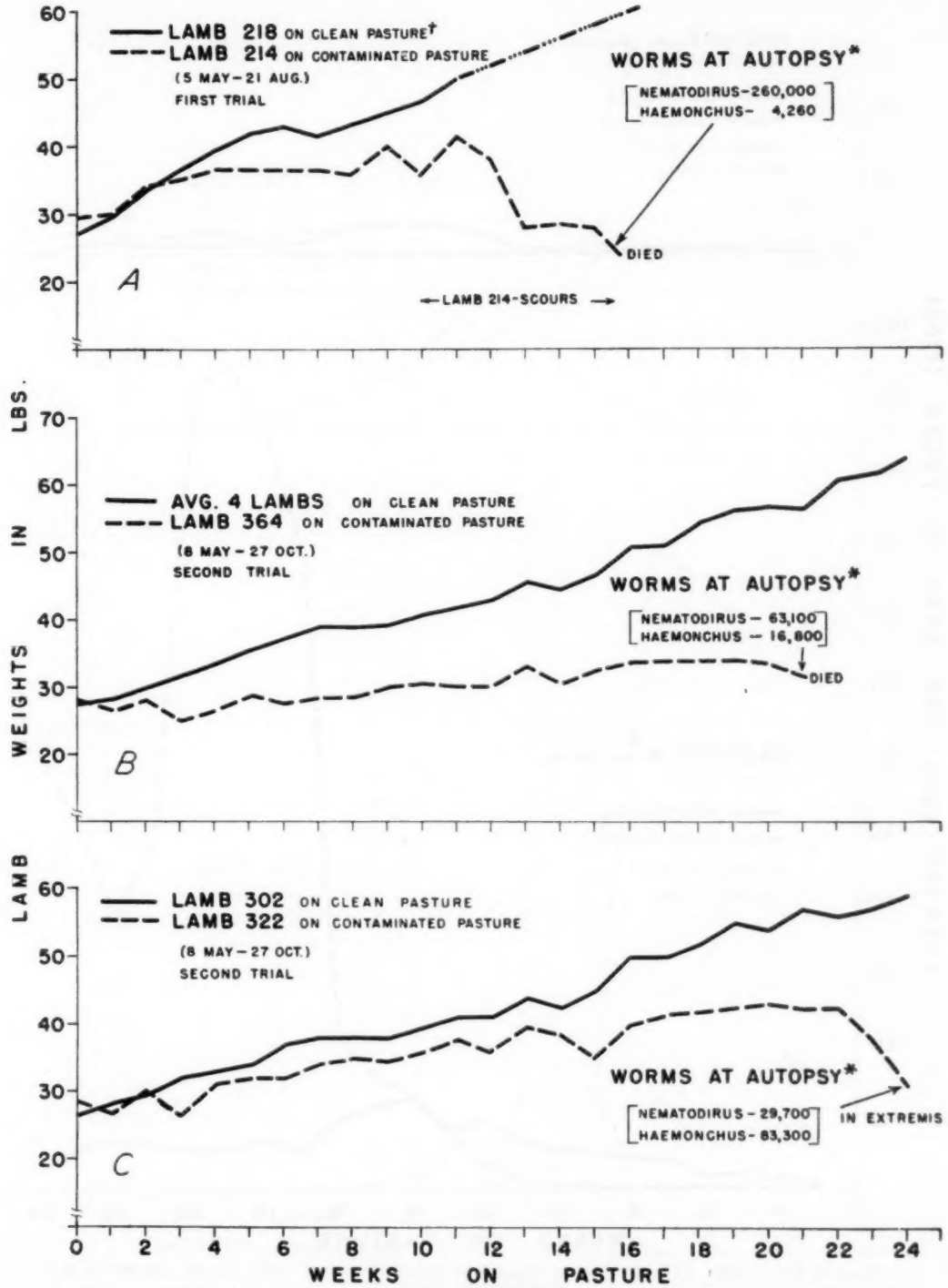
Second Trial.—In this trial, the medicated lambs (fig. 4 A) had lower fecal counts of *N. spathiger* and *H. contortus* eggs throughout the experiment than the comparable lambs on salt only (fig. 4 B). As in the first trial, the fecal egg count data showed the excellent survival of *N. spathiger* larvae and poor survival of *H. contortus* larvae over winter, particularly in the unmedicated pasture B lambs. Average egg counts were much lower for medicated, pasture A lambs than for comparable lambs during the first trial. The egg count data for the 2 lambs dying from parasitism on pasture B (fig. 5 A, B) showed a rapid increase of *N. spathiger* infections to a high level but a considerable delay in acquisition of heavy *H. contortus* infections. The lambs on clean pasture C developed light infections of *N. spathiger* and *H. contortus* during the latter weeks of the experiment, as a result of unavoidable contamination.

Lamb 322 (fig. 3 C, 5 B) developed diarrhea intermittently with mushy feces during the last three weeks of the experiment, and mushy feces were noted daily in lamb 364 (fig. 3 B) three weeks prior to its death. Feces were normal for the other 2 lambs on this pasture and those of groups A and C throughout the course of the experiment.

After twenty-four weeks on pasture, the total weight of the medicated lambs was 43.5 lb. greater than the total weight of those not medicated (table 1, second trial). The lambs on clean pasture C (fig. 3 B-solid line) gained 91 lb. more than the unmedicated lambs on pasture B and 47.5 lb. more than the medicated lambs on pasture A. Average weight gain per lamb for the 24-week period was group A, 24.5 lb.; group B, 16.8 lb.; and group C, 36.4 lb.

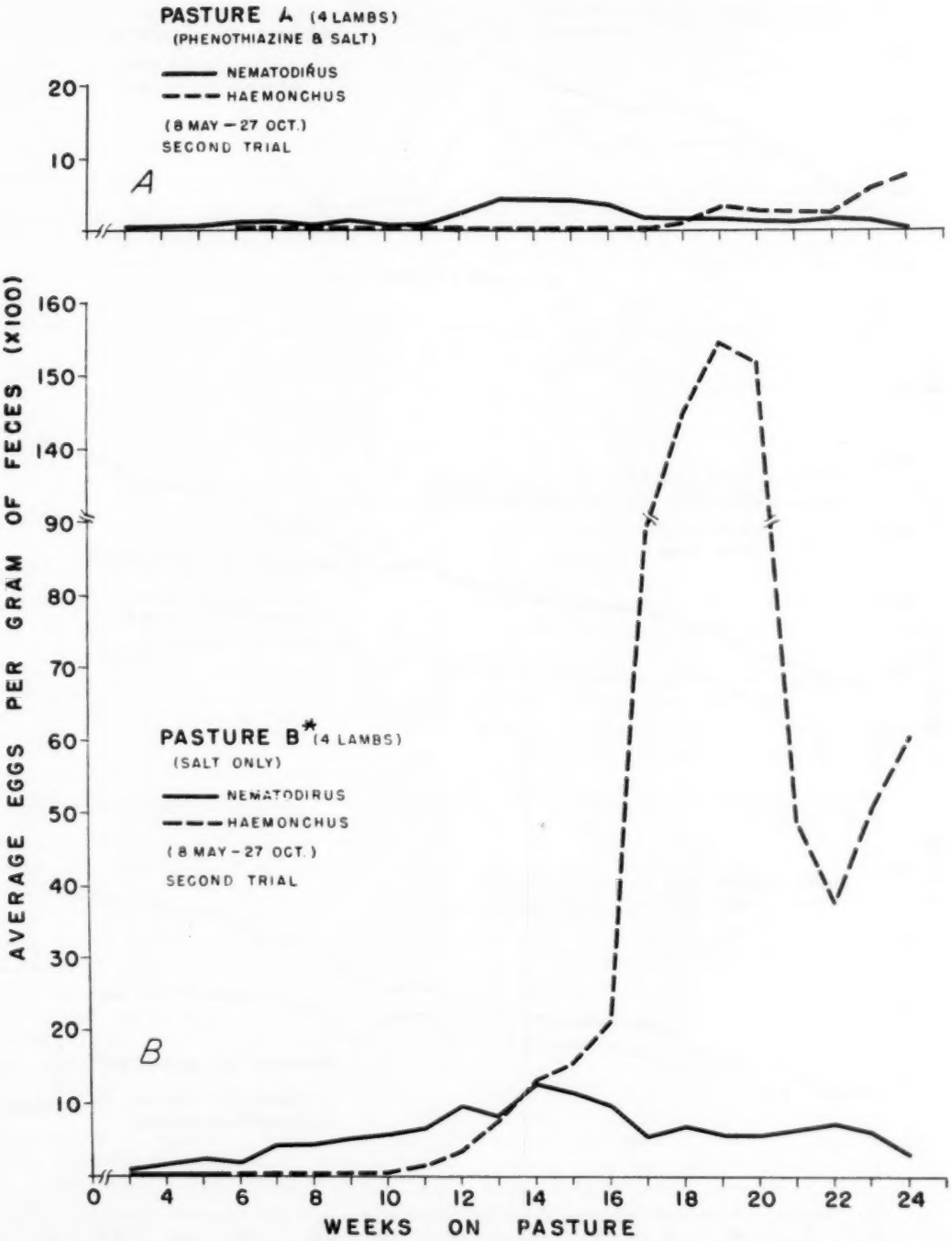
The lambs on pastures A and C showed no evidence of anemia throughout the experiment. Evidence of anemia began to appear in group B lambs during the twelfth week after which the anemia gradually became more acute. Lamb 364 died during the twenty-first week and lamb 322 was *in extremis* at the termination of the experiment; terminal hematocrit levels of these lambs were 9 per cent or less.

The postmortem worm counts obtained from the medicated and unmedicated lambs (table 2, second trial) showed the beneficial effect of the medication as in the



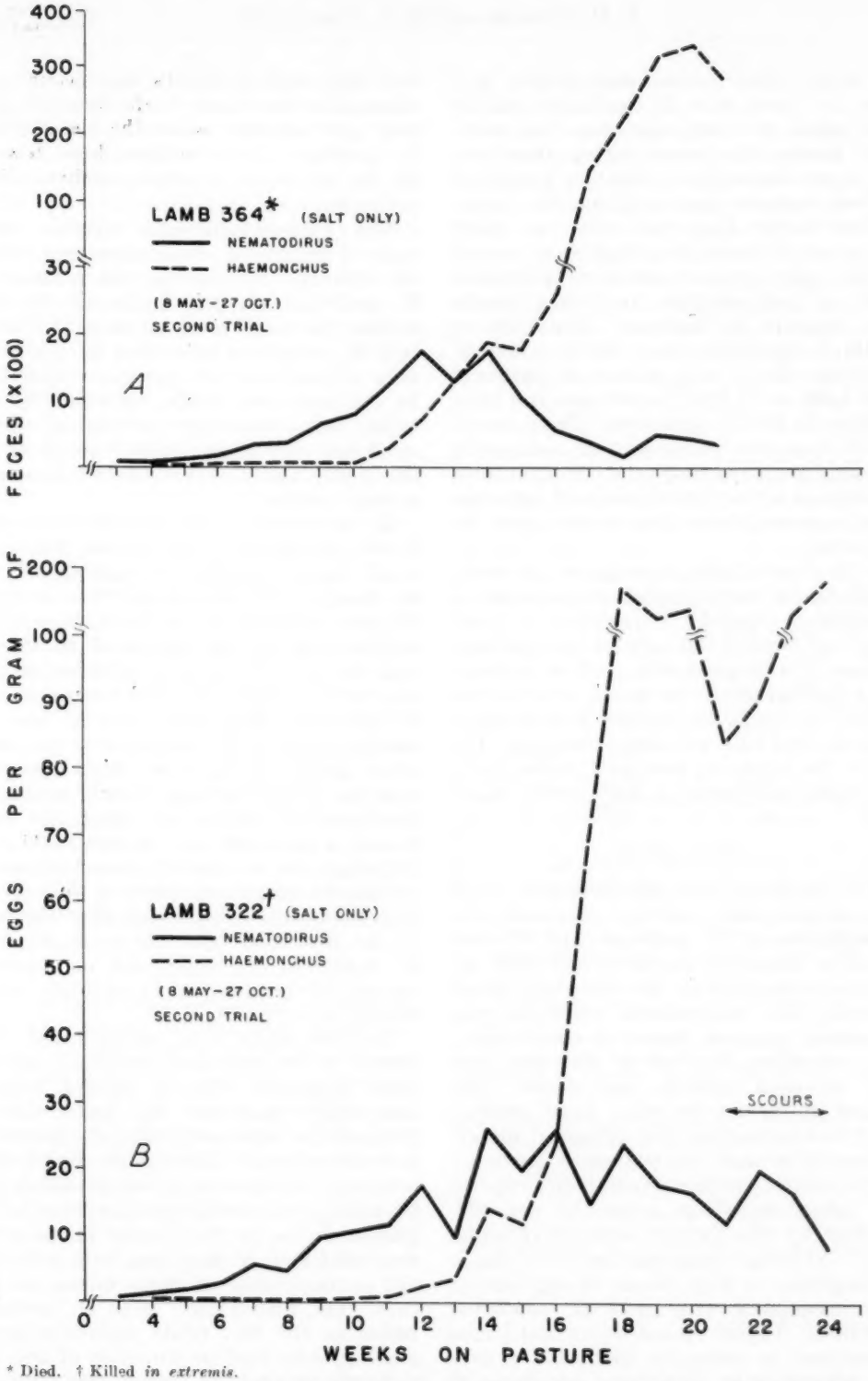
* Worms recovered postmortem from fatal cases inserted; worm burden of lambs on clean pasture not significant. † Lamb 218 on clean pasture only during eleven weeks, first trial.

Fig. 3—First and second trials, weight data of 3 fatal cases among unmedicated lambs compared with comparable lambs on clean pasture.



* On pasture B 1 lamb (364) died during twenty-first week and 1 lamb (322) was *in extremis* at end of experiment (fig. 5).

Fig. 4—Second trial, average weekly differential egg counts of lambs on contaminated pastures.



* Died. † Killed *in extremis*.

Fig. 5.—Second trial, weekly differential egg counts of 2 unmedicated lambs on contaminated pastures.

first trial. The unmedicated lambs harbored 3.6 times more *N. spathiger* and 30 times more *H. contortus* than the medicated lambs; the worm ratios, therefore, were approximately reversed in regard to the two parasite species in the two trials. Similar to the first trial, however, there was great variation in numbers of worms of both species recovered from individual lambs in both groups. Individual lambs from pasture A harbored from 650 to 11,800 *N. spathiger,* and 550 to 3,300 *H. contortus;* those from pasture B harbored from 4,300 to 63,100 *N. spathiger* and from 5,000 to 85,100 *H. contortus.* The percentage of immature worms was approximately the same as in the first trial. Insignificant numbers of a few other species of parasites were recovered from these lambs as in the first trial.

At the end of this experiment, carcasses of all lambs were graded antemortem as "choice," "good," "medium," "common," or "cull." The grades for lambs on pasture A were good and medium, averaging a half grade below good, whereas the grades for lambs on pasture B were good, medium, and cull, averaging common. The grades for lambs on pasture C were choice and good, averaging a half grade above good.

DISCUSSION

It is apparent from the data that a 1:9 phenothiazine-salt mixture reduced the depredations of *N. spathiger* and *H. contortus* in combined, massive, naturally acquired infections. In the two trials recorded, the unmedicated lambs on contaminated pastures acquired severe parasitic infections that led to diarrhea, anemia, retarded growth, and death. The treated lambs, on the other hand, showed much less parasitism and remained in better condition than the untreated lambs on contaminated pastures. *Nematodirus spathiger* infections which, normally, are self-limiting by the fourth week after exposure,[3] exhibited exacerbations and also a prolongation of high levels of egg output when associated with gross *H. contortus* infections. Taylor[6] pointed out that lambs maintained on adequate diet have a definite advantage in developing resistance to parasitic infections as compared to those having access to insufficient or inadequate forage. The trials discussed herein indicate that similar results may occur when animals harbor heavy infections of more than one parasite, since the resistance to *N. spathiger* seems to have been lowered by the presence of large numbers of *H. contortus.*

The phenothiazine-salt mixture seemingly produced a dual, beneficial effect: (1) directly, by limiting the numbers of *N. spathiger* and (2) indirectly, by controlling the deleterious influence of concurrent *H. contortus* infections on the resistance of lambs to *N. spathiger* infections. In the medicated lambs, in which *H. contortus* infections were controlled, the *N. spathiger* infections were comparatively low grade and followed their presumably normal course.

Better control of *H. contortus* was noted in the medicated lambs during the second trial. Since infective *N. spathiger* larvae are hardier and less susceptible to winter kill than infective *H. contortus* larvae,[2] the earlier peak of egg output of the former and the delayed build-up of infection with the latter species reported herein were to be expected. The relatively high egg production peak of *H. contortus* in the medicated lambs of the first trial, moreover, was due to the fact that 1 lamb exhibited a maximum of 6,512 e.p.g., while the other showed a maximum of less than 1,000 e.p.g. Although the amount of phenothiazine individually consumed was not determined, it is a plausible hypothesis that the lamb in the first trial with the unusually high *H. contortus* egg count did not consume enough of the chemical to establish anthelmintic benefits.

In both trials, total worm counts were higher in the untreated lambs on contaminated pastures, than in treated ones on comparable pastures. The lower ratio of *N. spathiger* recovered from the medicated and unmedicated lambs of the first trial as compared to the second was probably due, in part, to a greater consumption of the phenothiazine by the treated lambs of the first trial and, in part also, to a lower initial concentration of these worms on pasture. The vast difference in *H. contortus* ratios in the two trials indicates that a much greater pasture infection of this parasite was present the second year than during the first. Significant control, however, was achieved both years in the medicated groups.

In both trials, a slightly higher percentage of immature *N. spathiger* was recovered from the medicated lambs than from the unmedicated lambs. This may mean that fewer *N. spathiger* developed to maturity in these lambs because of the effect of the medication. On the other hand, higher percentages of immature *H. contortus* were recovered from the unmedicated lambs, which may be indicative of some efficacy of the drug against immature *H. contortus* under the conditions of this experiment. It may also be implied that these lambs were exposed to heavier contamination of this species and that many worms were spontaneously eliminated before or after reaching maturity, due probably to some acquired immunity response.

Proof of the beneficial action of the phenothiazine-salt mixture against *N. spathiger* was shown by the greater weight gains, the absence of diarrhea and death, and the suppression of the clinical aspects of nematodirosis in lambs on this regimen in comparison with lambs denied this protection.

Despite the significantly greater weight gains of the medicated lambs on contaminated pasture, as compared to their unmedicated controls, it is evident that the best gains were registered by the unmedicated lambs that were on comparatively parasite-free pastures. The advantages of the medication, although marked, seemed, therefore to be insufficient to offset the disadvantage of heavy exposure to parasitism.

SUMMARY

Two similar trials on pasture during two successive grazing seasons were carried out to test the efficacy of a 1:9 phenothiazine-salt mixture against combined natural infections of *Nematodirus spathiger* and *Haemonchus contortus*. A total of 12 lambs were employed in each trial. Lambs were grazed on parasite-contaminated pastures with one group receiving the medicated mixture, the other having access to salt only. A control group of comparable lambs was also maintained on a comparatively parasite-free pasture.

Lambs on medication consistently displayed lower degrees of parasitism, better weight gains, fewer abnormal stools, and higher hematocrit levels than untreated lambs on contaminated pastures. Furthermore, untreated lambs on comparatively parasite-free pasture made even greater weight gains than did the treated lambs on contaminated pastures. No fatalities occurred in either the medicated groups or in the untreated control group on clean pasture, whereas 2 died and another was killed *in extremis* in the unmedicated groups on contaminated pastures.

Weekly fecal egg counts indicated in some lambs with mixed infections that the *H. contortus* not only reversed the normal self-limiting course of *N. spathiger* infections but caused exacerbation and prolongation of nematodirosis. The results of these tests suggest that the phenothiazine-salt regimen is of some direct value in the control of *N. spathiger*, and particularly beneficial when coexistent *H. contortus* infections are present.

References

[1] Foster, A. O., Turner, J. H., Kates, K. C., Sinclair, L. R.: Control of Nematodirus Infections of Lambs by Free-Choice Administration of Phenothiazine. J. Parasitol., *38*, (1952): 11.

[2] Kates, K. C.: Survival on Pasture of Free-Living Stages of Some Common Gastrointestinal Nematodes of Sheep. Proc. Helminthol. Soc. Washington, *17*, (1950): 39-58.

[3] Kates, K. C., and Turner, J. H.: Experimental Studies on the Pathogenicity of *Nematodirus Spathiger*, a Trichostrongylid Parasite of Sheep. Am. J. Vet. Res., *14*, (1953): 72-81.

[4] Report of the Chief of the Bureau of Animal Industry, Agricultural Research Administration, U.S.D.A., (1948): 64.

[5] Swales, W. E.: Pasture Studies on Parasitic Diseases of Sheep in Eastern Canada. I. The Use of Phenothiazine-Salt Mixture During the Grazing Season. Canad. J. Comp. Med., *16*, (1952): 48-56.

[6] Taylor, E. L.: The Interaction of Nutrition and Parasitism with Special Reference to Parasitic Gastritis. Vet. Rec., *55*, (1943): 117-119.

[7] Turner, J. H.: Counting *Nematodirus Spathiger* Eggs in Sheep Dung. Proc. Helminthol. Soc. Washington, *18*, (1951): 132-135.

[8] Turner, J. H., and Colglazier, M. L.: Further Studies of the Value of Phenothiazine, Free-Choice, Against Mixed Infections of Nematodirus and Haemonchus in Lambs. J. Parasitol., *39*, (1953): 100.

Vaccination of Sheep with *Clostridium Perfringens* Type D Vaccines

R. C. PERCIVAL, V.M.D.; R. L. BURKHART, V.M.D.; M. S. COOPER, Ph.D.; F. V. MARTINI, B.S.

Pearl River, New York

Literature Cited

The history of vaccination of sheep against enterotoxemia has been reviewed in detail by Muth and Morrill,[1] Wilson and Miles,[2] and Whitlock and Fabricant.[3] It will suffice to state that Bennetts [4] and Gill [5] were among the first to report the value of formolized vaccine in protecting lambs against this disease.

Since the studies reported above, the vaccine has attained appreciable attention and world-wide use. As evidence of the demand for this product, there are about 12 producers in the United States. During the period of June 1952-June 1953, about 2½ million doses were produced.

It is not within the scope of this paper to consider the value of this type of vaccine under field conditions. Our objective was rather to study the troublesome local reactions produced by this vaccine with the hope of minimizing this important disadvantage without affecting the efficacy of the product.

Baldwin [6] pointed out that local reaction to vaccination may persist for thirty days, necessitating trimming the lesion from the carcass if the animal is marketed within that period. Our experience with the vaccine produced in our laboratories, as well as with others available in the United States, indicates that local reactions are quite persistent and confirm Baldwin's work.

The vaccines presently available commercially are alum-precipitated, formolized whole cultures. The following studies were carried out to observe, under controlled conditions, the severity and frequency of local reactions produced by this type of product, and the effects of omitting alum on the occurrence of reactions and the immune response.

From the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The authors are indebted to Dr. Frank Wilcoxon for statistical analyses, and to Mr. R. H. Katt and Mr. S. C. Griffith for technical assistance.

The Effect of Alum Precipitation on the Severity of Local Reactions to Clostridium Perfringens Type D Vaccine

Materials and Methods.—A small lot of vaccine was produced according to the procedure described by DeMello and his associates.[7] Half of the material was precipitated with 0.75 per cent K $Al(SO_4)2 \cdot 12H_2O$ while half was not alum precipitated (anaculture).

Vaccination of Lambs.—Twenty-four lambs, about 6 to 8 months old, were divided into two groups of 12 each. In one group each received 5 ml. of anaculture; in the other each received 5 ml. of alum-precipitated vaccine. All injections were made subcutaneously, just back of the elbow on the left side, on June 22, 1953. All animals were observed for twenty-three days.

Table 1 lists the size of reactions observed. In the group receiving anaculture, 11 of 12 lambs showed negative or minimal reactions at the end of the observation period, while all lambs injected with alum-precipitated vaccine showed an appreciable swelling at this time.

Local Reactions Produced by Several Combinations of Components in Alum-Precipitated Clostridium Perfringens Type D Vaccine

Materials and Methods.—A small lot of vaccine was prepared. During the preparation, various samples were removed to determine the effects of the various components on local reactions.

The culture medium regularly used for the production of vaccine was prepared (sample 1). The medium was inoculated with *Cl. perfringens* type D organisms and incubated. After the required growth period, formalin was added (sample 2). Sample 2 was divided and half was alum precipitated (sample 3). A part of the formolized whole culture was then centrifuged for one hour at 2,500 r.p.m. The supernatant was removed as toxoid (sample 4). Sample 4 was divided and to half was added 0.75 per cent alum (sample 5). The sediment from centrifugation was washed several times with physiological saline and was resuspended to the original volume with 0.75 per cent alum solution (sample 6).

Sample 2, formolized whole culture, was assayed and found to contain 0.48 per cent formalin. Formalin solution in distilled water at 0.48 per cent concentration was made (sample 7). A 0.75 per cent alum solution was also prepared (sample 8).

Injection of Lambs.—Twenty-five lambs, about 6 to 8 months of age, were used for this study. Each animal was clipped on both sides. Four different samples were injected on each side at marked sites located equidistantly in a 6- by 8-in. area. Five milliliter of each preparation was injected subcutaneously.

Results.—The animals were injected on Oct. 15, 1953 and were observed for seventy-four days. The severity of local reaction was determined by actual measurement. Due to the volume of such data collected, and since it appeared that frequency of occurrence was more notable than extent of reaction, this report will stress the latter observations.

Local reactions occurred as early as the first day following inoculation, but no new reactions appeared after the eighth day. Usually, reactions began to subside about fifteen days postinoculation but persisted for varying periods, not appearing to fall into any specific pattern. In no case did the single components—complete medium, formalin, or alum—produce a measurable reaction in any of the animals. Samples containing alum (3, 5, and 6) caused a considerably greater number of reactions than those without alum (2 and 4) as shown in table 2. By the use of the Mainland tables,[8] it was determined that differences in frequency of occurrence of these reactions were statistically significant.

THE EFFECT OF ALUM PRECIPITATION ON INTENSITY AND DURATION OF ANTITOXIN RESPONSE TO VACCINATION WITH CLOSTRIDIUM PERFRINGENS TYPE D VACCINE

Materials and Methods.—A small lot of vaccine was produced according to the procedures described by DeMello.[7] The formolized whole culture was tested to determine the concentration of toxoid present according to the method of Baldwin, Frederick, and Ray,[9] using an indicator dose of 5 m.l.d. The vaccine was found to contain between 200 and 300 combining units or between two and three times the minimum potency required by the U.S.-D.A., Animal Disease Eradication Branch. Half of the material was precipitated with 0.75 per cent $KAl(SO_4)2 \cdot 12H_2O$ and half was not alum precipitated (anaculture).

Vaccination of Lambs.—Twenty-four lambs, 6 to 8 months of age, were divided into two groups of 10 each, and one group of 4 uninoculated controls. One group of 10 received 5 ml. each of anaculture, while the other group received 5 ml. each of alum-precipitated vaccine. All injections were made subcutaneously just behind the right elbow, on Aug. 10, 1953. Test bleedings were taken from individual animals just prior to vaccination and 3, 9, 15, and 18 weeks postvaccination. The antitoxin titers were assayed in the following manner.

Dried *epsilon* toxin was reconstituted and activated with trypsin. This solution was titrated in mice to determine m.l.d./ml. present. The antitoxin unit of Baldwin[9] was used; namely, the smallest amount that will neutralize 15 m.l.d. of activated toxin. Toxin-antitoxin mixtures were made and held at room temperature for one hour before intravenous injection into mice. Serums were combined with toxin of 60 m.l.d./ml. potency.

TABLE 1—Effect of Alum Precipitation on Severity of Local Reactions

Sheep (No.)	Vaccine	Vaccinated (date)	Local reaction (inches)			
			6/26	6/29	7/7	7/15
1169	Alum	6/22	1 x ¼	1 x ½	1 x ½	1 x ¼
1170	Alum	6/22	1 x ½	1 x ¼	1 x ½	1 x ½
1171	Alum	6/22	Neg.*	1 x ½	1 x ¾	1 x ½
1172	Alum	6/22	Neg.	Neg.	1 x ¾	1 x ½
1173	Alum	6/22	1 x ½	1 x ½	1 x ½	1 x ½
1174	Alum	6/22	1 x ½	1 x ¾	1 x ½	1 x ¼
1175	Alum	6/22	1 x ¼	1 x ½	1 x ½	1 x ¼
1176	Alum	6/22	1 x ½	1 x ½	1 x ¾	1 x ½
1177	Alum	6/22	1 x ½	1 x ¾	1 x ½	1 x ½
1178	Alum	6/22	1 x ¼	1 x ½	1 x ½	1 x ½
1179	Alum	6/22	1 x ½	1 x ½	1 x ½	1 x ¼
1180	Alum	6/22	1 x ¼	1 x ¾	1 x ½	1 x ¼
1181	Anaculture	6/22	1 x ¼	Sl.**	Neg.	Neg.
1182	Anaculture	6/22	Neg.	Neg.	Neg.	Neg.
1183	Anaculture	6/22	1 x ¼	Neg.	Neg.	Neg.
1184	Anaculture	6/22	¾ x ¼	1 x ¼	1 x ¼	Sl.
1185	Anaculture	6/22	1 x ¼	Sl.	1 x ¼	1 x ¼
1186	Anaculture	6/22	Neg.	1 x ¼	1 x ¼	Sl.
1187	Anaculture	6/22	Neg.	Neg.	Sl.	Sl.
1188	Anaculture	6/22	1 x ¼	1 x ½	1 x ½	Sl.
1189	Anaculture	6/22	Sl.	Sl.	Neg.	Neg.
1190	Anaculture	6/22	Sl.	Sl.	Sl.	Sl.
1191	Anaculture	6/22	Neg.	Neg.	Neg.	Neg.
1192	Anaculture	6/22	1 x ½	¾ x ½	1 x ½	Sl.

No reactions were noted one day postvaccination; * negative; ** slight.

The injection dose for mice was always 0.5 ml. and mice always received 15 m.l.d. of toxin in this dose. It is, therefore, obvious that this test can not detect less than 4 units of antitoxin per milliliter.

TABLE 2—Number of Reactions to Various Preparations

Sample (No.)	Preparation	Reacting/ total (No.)	Reacting (%)
1	Complete medium	0/25	0
2	Formolized whole culture	4/25	16
3	Alum-precipitated, formolized whole culture vaccine	19/25	76
4	Toxoid	7/25	28
5	Toxoid containing 0.75 % alum	20/25	80
6	Washed cells containing 0.75 % alum	22/25	88
7	0.48 % formalin	0/25	0
8	0.75 % alum	0/25	0

Results.—The results of this study are listed in table 3. The lambs receiving anaculture did not show lower antitoxin levels than those receiving the alum-precipitated vaccine, nor was there any difference observed in the maintenance of antitoxin levels.

DISCUSSION

The value of aluminum adjuvants in increasing antigenicity of toxoids has been well established by Glenny and his associates.[10,11] It is also well established that these adjuvants may appreciably increase the local reaction produced by the vaccine. In spite of the generally accepted feeling that some degree of inflammation is desirable for an enhanced immune response, a swelling at the injection site which persists for many weeks may seriously limit the usefulness of such a product in spite of its effectiveness.

The use of alum as an adjuvant for this vaccine was reported by Buddle.[12] It seems that his paper is the basis for the inclusion of alum in present vaccines. However, it is our feeling that Buddle's studies involved an appreciable number of uncontrolled variables making the conclusion that alum actually improved the value of the vaccine somewhat tenuous.

In our studies, we have presented some evidence that alum may appreciably increase the local reaction caused by the enterotoxemia vaccine as prepared by our procedure. In a more detailed study involving several combinations of components of the final alum-precipitated product, we have demonstrated that alum alone will not produce a lesion but does have this effect in the presence of whole culture, toxoid, or cells separated from formolized whole culture. This interaction seems to make the removal of cells of questionable value in decreasing the severity of the local reaction.

Under the conditions of our experiment, we have demonstrated that alum can be

TABLE 3—Effect of Alum Precipitation on Duration of Antitoxin Response

Sheep (No.)	Pre-injection titer	Vaccine	Postvaccination titer			
			3 weeks	9 weeks	15 weeks	18 weeks
1807	<4	Anaculture	4	<4	<4	<4
1806	<4	Anaculture	32	32	12	32
1815	<4	Anaculture	32	32	12	12
1812	<4	Anaculture	8	<4	<4	<4
1810	<4	Anaculture	<4	<4	<4	<4
1819	<4	Anaculture	8	<4	<4	<4
1814	<4	Anaculture	4	<4	<4	<4
1817	<4	Anaculture	32	8	<4	<4
1802	<4	Anaculture	<4	<4	<4	<4
1816	<4	Anaculture	4	<4	<4	<4
1803	<4	Alum	4	<4	<4	<4
1821	<4	Alum	32	8	<4	<4
139	<4	Alum	8	<4	<4	<4
1808	<4	Alum	24	8	4	8
1809	<4	Alum	32	8	<4	<4
1805	<4	Alum	16	<4	<4	<4
1823	<4	Alum	8	12	<4	<4
1820	<4	Alum	16	<4	<4	<4
1825	<4	Alum	Died			
1824	<4	Alum	<4	<4	<4	<4
1804	<4	Controls	<4	<4	<4	<4
1813	<4	Controls	<4	<4	<4	<4
1818	<4	Controls	<4	<4	<4	<4
1811	<4	Controls	<4	<4	<4	<4

omitted from the vaccine without having a measurable effect on the antitoxin response. We believe that the removal of alum would result in a more useful product since the troublesome persistent lesions can, in most cases, be avoided, while a durable immunity equal to that produced by the alum-precipitated product is induced. It is important to point out that this change has been carried out with good results by Alexander [13] in the Union of South Africa.

SUMMARY

1) The presence of alum in *Clostridium perfringens* type D vaccine was shown to increase the severity and persistence of local reactions after vaccination.

2) Alum alone produced no reactions and anaculture only a minimal response. However, alum in combination with toxoid or washed cells produced a reaction equal to that of the alum-precipitated vaccine.

3) No significant difference in the intensity or duration of the immune response (as determined by antitoxin levels) was observed when alum was omitted from the vaccine.

References

[1] Muth, O. H., and Morrill, D. R.: Control of Enterotoxemia (Pulpy Kidney Disease) in Lambs by the Use of Alum-Precipitated Toxoid. Am. J. Vet. Res., *7*, (1946): 355.

[2] Wilson, G. S., and Miles, A. A.: Topley and Wilson's Principles of Bacteriology and Immunity. 3rd ed. The Williams & Wilkins Co., Baltimore, *2*, (1946): 1782.

[3] Whitlock, J. H., and Fabricant, J.: The Use of *Clostridium Welchii* Type D Anaculture in the Prevention of Overeating Disease (Enterotoxemia) in Sheep. Cornell Vet., *37*, (1947): 211.

[4] Bennetts, H. W.: Infectious Enterotoxemia (The So-called Braxy-like Disease) of Sheep in Western Australia. Council Sci. & Indust. Res. Australia, Bull. 57, (1932): 72.

[5] Gill, D. A.: Pulpy Kidney Disease of Lambs. New Zealand J. Agric., *45*, (1932): 332.

[6] Baldwin, E. M.: Enterotoxemia of Sheep and Its Control. Vet. Med., *45*, (1950): 473.

[7] DeMello, G. C., Clemente, J., Kiser, J. S., and Williams, J. H.: The Preparation and Testing of *Clostridium Perfringens* Type D Bacterins. Proc. U. S. Livestock San. A., (1952): 295.

[8] Mainland, D., and Murray, I. M.: Tables for Use in Fourfold Contingency Tests. Science, *116*, (1952): 591.

[9] Baldwin, E. M., Frederick, L. D., and Ray, J. D.: The Control of Ovine Enterotoxemia by the Use of *Clostridium Perfringens* Type D Bacterin. Am. J. Vet. Res., *9*, (1948): 296.

[10] Glenny, A. T., Pope, C. G., Waddington, H., and Wallace, U.: The Antigenic Value of Toxoid Precipitated by Potassium Alum. J. Path. and Bact., *29*, (1926): 38.

[11] Glenny, A. T.: Insoluble Precipitates in Diphtheria and Tetanus Immunization. Brit. M. J., *2*, (1930): 244.

[12] Buddle, M. B.: The Production of Immunity Against *Clostridium Welchii* Type D (Enterotoxemia). Austral. Vet. J., *17*, (1941): 3.

[13] Alexander, R. A.: Personal communication.

The Microscopic Anatomy of the Digestive Tract of *Sus Scrofa Domestica*

MARGARET W. SLOSS, D.V.M., M.S.

Ames, Iowa

IN THIS STUDY, the entire digestive tracts, with the exception of the teeth, pancreatic glands, and livers of 4 swine were used. When conclusions could not be drawn from this material, sections from other cadavers were taken to supplement the study. In all, tissues from portions of 15 pigs were examined. Four complete tracts were taken; one from a newborn pig, one from a 35-lb. pig, one from a pig weighing approximately 125 lb., and one from a pig weighing approximately 225 lb. The entire discussion is given from the tissues of the pig weighing 125 lb., unless definite mention is made concerning the age. The ordinary methods of fixation, embedding, and staining were employed.

ANATOMY OF THE DIGESTIVE TRACT

The Lips.—Six to 25 layers of cells, exclusive of the cornified layer, cover the cutaneous surface of the lips. The stratified squamous epithelium on the oral surface is 8 to 50 cells thick. Prominent intercellular bridges are observed.

The corium lying deeply to the cutaneous epithelium is made up of a comparatively dense white fibrous connective tissue and a few elastic fibers. Penetrating this corium from the muscle layer are striated muscle fibers reaching to the border of the epithelium. Smooth muscle fibers are seen in connection with the hair follicles. Adipose and areolar as well as white fibrous connective tissue separate the muscle bundles which run through the corium. Sudoriparous glands, blood vessels, and nerves are very abundant between the muscle bundles. The labial muscles separate the corium of the external surface of the lip from the corium of the internal surface. The oral corium is thicker but less dense than the cutaneous corium. The white fibrous connective tissue of the oral corium contains a great many elastic fibers near the muscle layer, but near the epithelial layer there are relatively few.

The labial glands, which are found in the oral corium, are present only in the commissural third of the upper lip (fig. 1) but they can be demonstrated in the commissural half of the lower lip (fig. 2). These glands are tubuloacinar containing serous acini and mucous acini with serous demilunes, there being a predominance of the former in most of the lobules. The serous demilunes are very large.

The Cheeks.—The mucosa of the cheeks is smooth and lined by a many-layered stratified squamous epithelium. The mucosa presents a papilla which marks the entrance for the ductus parotideus near the second superior molar tooth. Goblet cells are present in the duct and in the squamous epithelium near the duct opening on to the cheek (fig. 3). The corium of the cheek, of loose white fibrous connective tissue, contains a few elastic fibers. Bundles of striated muscle extend almost to the base of the epithelium. Tubuloacinar glands, glandulae buccales, are intermingled with fibers of the buccinator muscle. Predominating are mucous acini with serous demilunes but a few acini are serous and a few are mucous. Serous acini are more numerous in the inferior buccal gland. Larger amounts of white fibrous connective tissue separate the acini of these glands than is true for the submaxillary gland. Goblet cells increase in numbers in the ducts as the buccal surface is approached.

The Hard Palate.—The corrugated hard palate of swine is composed of fatty cushions embedded in connective tissue and covered on the oral surface by stratified squamous epithelium of 6 to 40 cells in depth (fig. 4). The surface cells are keratinized and only flattened nuclear remnants are seen. Intercellular bridges are very distinct in all layers except those of the keratinized portion. Large cells (fig. 5) are observed, individually or in nests, in

From the Department of Veterinary Anatomy, Veterinary Division, Iowa State College, Ames. For the entire work and references see "The Microscopic Anatomy of the Digestive Tract of *Sus Scrofa Domestica*," Master's Thesis, Library, Iowa State College, 1932.

the epithelium extending between the connective tissue papillae. Their cytoplasm remains clear while that of the other epithelial cells appears granular. Their large vesicular nuclei are round or bean-shaped and almost fill the cells. The chromatin granules in these nuclei are not evenly distributed, as is true in other epithelial cells, portions appearing almost clear. Presumably these are tactile cells.

The connective tissue, immediately beneath the basement membrane, is composed of dense white fibrous connective tissue fibers and numerous fine yellow elastic fibers. The connective tissue papillae are richly supplied with capillaries. In the

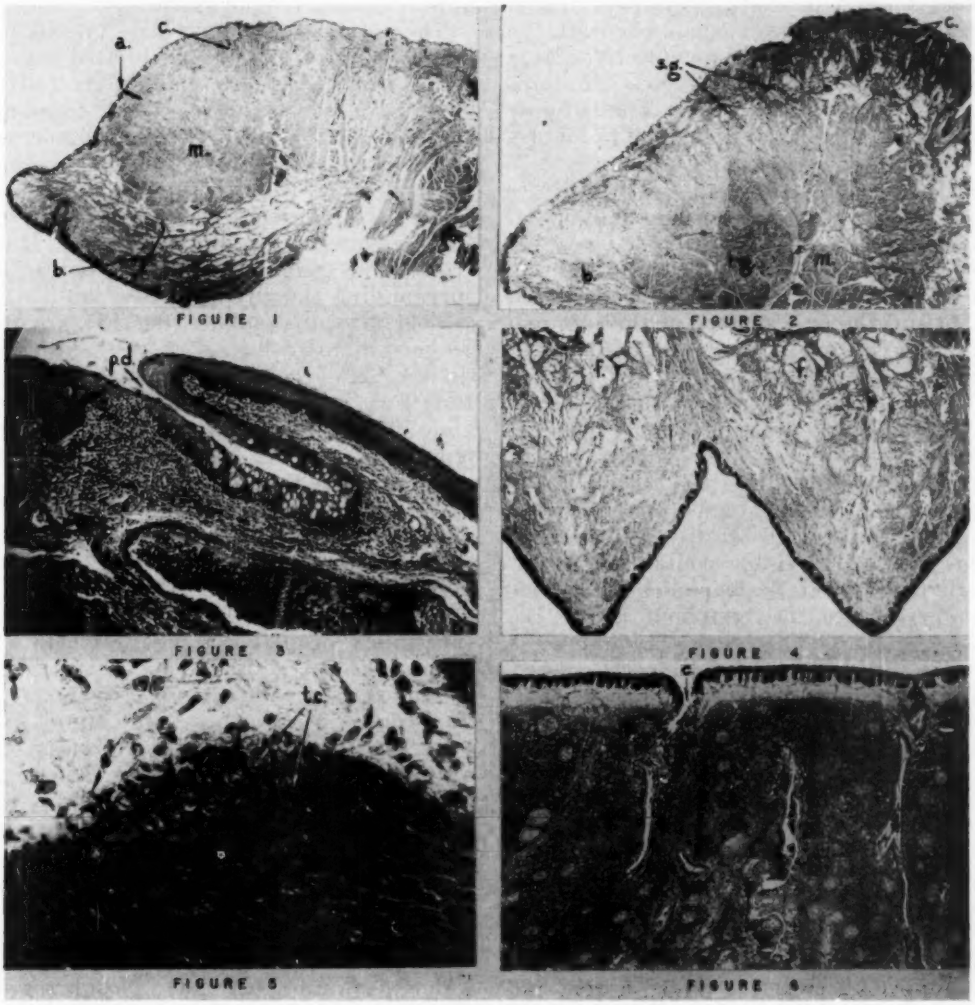


Fig. 1—Upper lip, showing relative thickness of the corium of the skin (a) and of the mucosa (b), a hair follicle (c), and muscles (m) but no labial glands. x 3.5.

Fig. 2—Lower lip, showing (a), (b), (c), and (m) as in figure 1, also sudoriferous glands (s.g.) and masses of labial glands (l.g.). x 3.5.

Fig. 3—The parotid duct (p.d.) perforating the cheek tangentially. Beadlike goblet cells shown in the buccal stratified squamous epithelium. x 50.

Fig. 4—Hard palate. Fat pads (f) are present underneath the ridges. x 9.

Fig. 5—Hard palate. Large cells in epithelium are tacticle cells (t.c.). x 270.

Fig. 6 — Palatine tonsil. Crypts (c) lined by stratified squamous epithelium extend through the lymphoid cells to the corium. x 12.

large ridges forming the corrugations of the palate are areas of connective tissue differing in staining properties from either white fibrous or yellow elastic connective tissue. In these areas are fat cells in various stages of metamorphosis. An arteriole can be seen in the center of each area.

Above the level of the corrugations, the connective tissue is areolar in type and possesses large deposits of adipose tissue, which appear to form cushions beneath the ridges of the palate. The connective tissue beneath the palate depressions contains little or no adipose tissue. The adipose tissue appears separated, similar to lobules of glands, by strands of dense white fibrous connective tissue. The hard palates of the specimens studied are glandless and contain no muscle fibers.

The Palatine Tonsils.—The palatine tonsils mark the boundary between the hard palate and the oral surface of the soft palate. Nine to 25 layers of cells form the stratified squamous covering. Crypts lined with stratified squamous epithelium (fig. 6) extend from the surface through the lymphoid tissue, reaching almost to the submucosa beneath the tonsillar mass. Between the epithelial covering and the lymphoid tissue is a stratum of white fibrous connective tissue containing a few elastic fibers. This accompanies the epithelial crypts into the lymphoid mass. Beneath the lymphoid tissue, the submucosa is composed of dense white fibrous connective tissue and large elastic fibers.

The Soft Palate.—On the oral surface of the soft palate, the stratified squamous epithelium is 8 to 30 cells thick (fig. 7), and on the aboral surface it is 5 to 18 cells in depth (fig. 8) changing gradually, as it approaches the nasal cavity, into pseudostratified ciliated columnar epithelium (fig. 9) at approximately the middle of the dorsal surface of the soft palate.

The submucosa is composed of dense white fibrous connective tissue, coarse and fine elastic fibers and patches of lymphoid tissue, which form nodules in some instances. Between the two submucosas lie the palatine muscles among which are located large numbers of tubuloacinar mucous glands, which form a complete layer on the ventral side of the palate. Their ducts contain a profusion of large goblet cells.

The Tongue.—The striated muscle of the tongue runs in various directions and between the bundles is loose white fibrous connective tissue containing elastic fibers, adipose tissue, tubuloalveolar glands, blood vessels, and nerves. The dorsal stratified squamous epithelium is 20 to 50 layers in depth (fig. 10). Fungiform, filiform, circumvallate, and foliate papillae appear on the tongue surface.

The two circumvallate papillae are the largest (fig. 11) and are located on the dorsal surface near the base of the tongue. They have true connective tissue cores, and they do not project above the tongue surface. Large taste buds (fig. 12) appear in the epithelium which covers the lateral surface of the papillae. They extend from the surface into the membrana propria. Ducts from serous glands empty into the trenches surrounding the circumvallate papillae.

The filiform papillae (fig. 13) are projections of many layers of epithelium and do not possess connective tissue cores. The fungiform papillae (fig. 14) do not project above the tongue surface, but they have true connective tissue bases. Taste buds are demonstrable on the dorsal surface of these papillae. The foliate papillae (fig. 15) occur as elongated narrow folds on the lateral surfaces of the tongue near the base and contain a few taste buds.

Beneath the epithelium of the tongue is the tunica propria composed of white fibrous connective tissue and elastic fibers. There is more elastic connective tissue in the papillae bases than in the tunica propria in general. The muscle bundles of the tongue follow three general directions, longitudinal, transverse, and vertical. The muscle is inserted in the tunica propria extending almost to the epithelial layer.

Tubuloacinar glands are distributed among the muscle bundles. Three types of secreting acini are observed; purely mucous, purely serous, and mucous acini with serous demilunes (fig. 16). No glands are found near the tip of the tongue but, along the lateral margins, numerous purely mucous acini occur. Near the circumvallate and foliate papillae the acini are largely of the serous type; although a few purely mucous and mixed acini can be seen. Numerous nerve bundles are distributed throughout the tongue, being especially prominent near the circumvallate papillae.

The Salivary and Accessory Salivary Glands.—Salivary and accessory salivary glands are of three types—(*a*) the parotid glands and the small glands located near the circumvallate and foliate papillae of the tongue have purely serous acini; (*b*) the glands of the soft palate and those found along the lateral margins of the tongue are mucous in type; and (*c*) the mandibular, sublingual, buccal, labial, and some of the lingual glands have mixed acini.

The Parotid Gland.—The parotid gland is the tubuloacinar type composed entirely

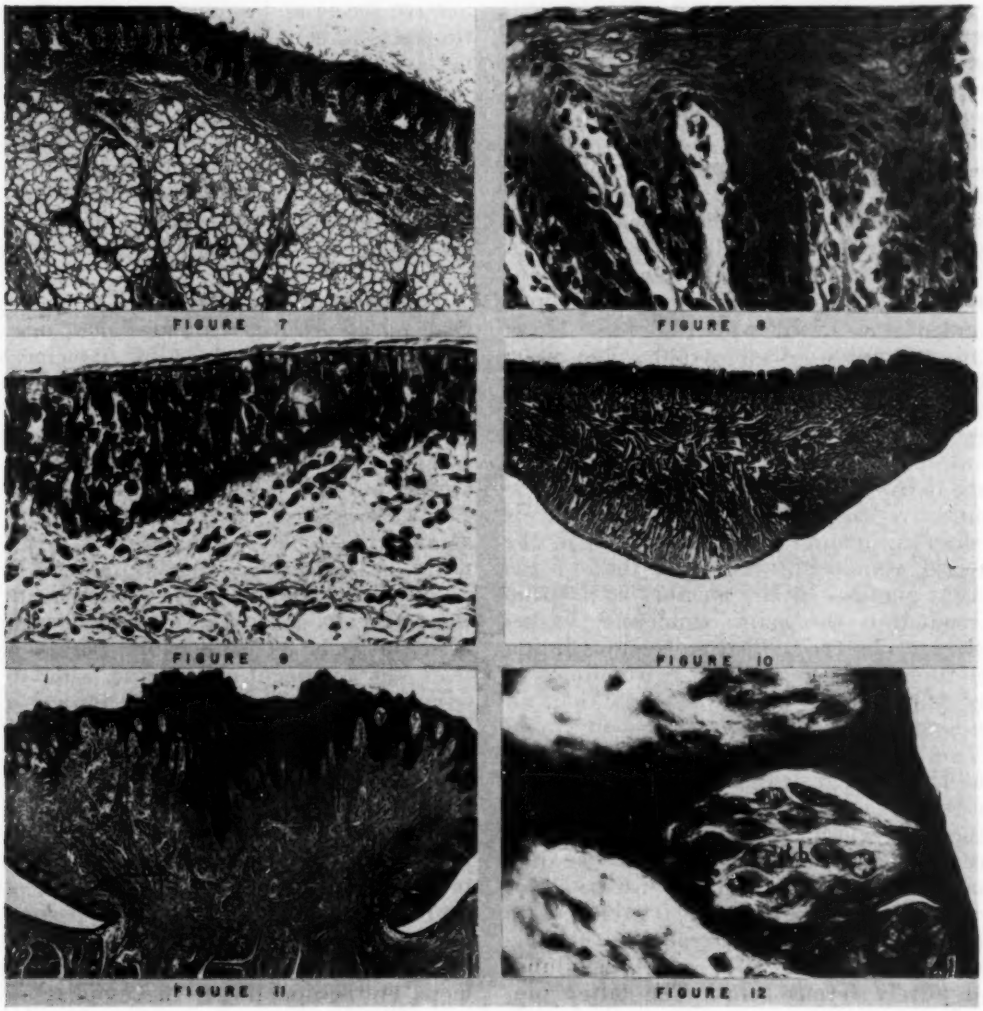


Fig. 7—Soft palate, oral surface. Mucous glands (m.g.) form almost a complete layer. x 22.5.

Fig. 8—Soft palate aboral surface. Squamous epithelium of the oral aspect. x 270.

Fig. 9—Soft palate. Pseudostratified ciliated columnar epithelium of the nasopharyngeal aspect. x 270.

Fig. 10—Tip of tongue. Stratified squamous epithelium covering all surfaces. Filiform and fungiform papillae on dorsal and lateral aspects. No glands in tip of tongue. x 3.5.

Fig. 11—Circumvallate papilla. Numerous taste buds (t.b.) shown along crypts. Abundance of nerve and gland tissue at base of papilla. x 19.

Fig. 12—Taste buds (t.b.). Buds extend from surface through epithelium into the tunica propria or corium. x 600.

of serous acini (fig. 17). The cells appear granular, although some of them are almost clear due probably to the solubility of the cell content to some chemical used in the fixing process. The acini are not separated by strands of connective tissue as they are in the mandibular gland. The lobules, on the other hand, are separated by greater amounts of white fibrous connective tissue, elastic fibers, and adipose tissue than is true for the mandibular gland. The parotid duct (fig. 3), which opens by means of a small papilla on the buccal mucosa, is lined by a several-layered cylindrical epithelium in which great numbers of exceptionally large goblet cells appear. These goblet cells appear in some instances in the basal-cell layer.

The Mandibular Gland.—The mandibular gland is a compound tubuloacinar gland of the mixed type (fig. 18). Three kinds of acini are demonstrable. The interlobular white fibrous connective tissue contains numerous elastic fibers. The very large demilunes on nearly all the mucous acini characterize this gland and differentiate it from the other salivary glands. The connective tissue separating the acini is greater in amount than that found in the parotid glands but less than that of the buccal glands. In the membrana propria, surrounding the acini, numerous basket cells are observed. The epithelium lining the ducts is similar to that of the ducts of any tubuloacinar gland, except that an abundance of goblet cells is present.

The Sublingual Gland.—The sublingual gland is divided definitely into a large anterior portion and a small posterior portion. The posterior portion empties by means of one duct, which joins the duct from the submaxillary gland, just posterior to its entrance into the buccal cavity. Two types of acini are demonstrable in this portion, mucous acini with serous demilunes and purely serous acini. The latter predominate.

The anterior portion is elongated and narrow, almost almond shaped, and appears to be divided into superior and inferior parts by the ducts of the posterior portion and of the mandibular gland. The superior part of the anterior portion has entire lobules comprised, for the most part, of purely serous acini, although a few mucous acini with demilunes can be observed. Other lobules contain only mucous acini with demilunes. The demilunes in this part of the gland are smaller than those in the mandibular gland (fig. 19). The inferior part of the anterior portion appears to be purely mucous in type, but very small demilunes are demonstrable on some of the mucous acini (fig. 20). The lobules of the gland are separated by white fibrous connective tissue and a large number of elastic fibers. There is considerably more connective tissue in this gland than is found in the mandibular gland.

The Pharynx.—The histology of the pharynx is similar to that of the pharyngeal pouch described below. The muscularis mucosae is absent and the tunica propria is comprised of white fibrous connective tissue containing coarse elastic fibers.

The Pharyngeal Pouch.—The pharyngeal pouch or diverticulum pharyngeum, an anatomical oddity peculiar to swine, situated in the posteriodorsal part of the pharynx between the ventral straight muscles of the head and the origin of the esophagus, is lined by stratified squamous epithelium of 5 to 15 cells in thickness (fig. 21). The tunica propria of white fibrous connective tissue and short elastic fibers contains numerous mucous glands and patches of lymphoid tissue. A few acini of these mucous glands have small demilunes of serous cells, and other acini possess cells near the basement membrane, similar to oxyntic cells in their reaction toward acid dyes. These delomorphous cells are larger than basket cells and smaller than the serous cells of the demilunes. Glands are also observed deeply embedded in the connective tissue separating the bundles of fibers of the striated muscle layer. Deeply to the muscle layers lies areolar connective tissue in which areas of adipose tissue can be demonstrated.

The Esophagus.—The stratified squamous epithelium lining the esophagus increases in thickness from 10 to 40 cells in the oral portion to 35 to 80 cells at the cardiac end. The muscularis mucosae appears in the esophagus first in the region opposite the cricoid cartilage. It is composed of an inner circular and an outer longitudinal layer of smooth muscle. These muscle layers do not form complete layers around the tube until near its middle.

The tunica propria of the esophagus has considerably more white fibrous and elastic

fibers than are found in the pharynx. Numerous, large tubuloacinar mucous glands are observed, appearing to be direct continuations of those found in the pharynx and pharyngeal pouch. However, these glands are not intermingled with the striated muscle of the tunica muscularis, as is the case in the pharynx and pharyngeal pouch. In the initial part of the esophagus, the mucous glands form two bands

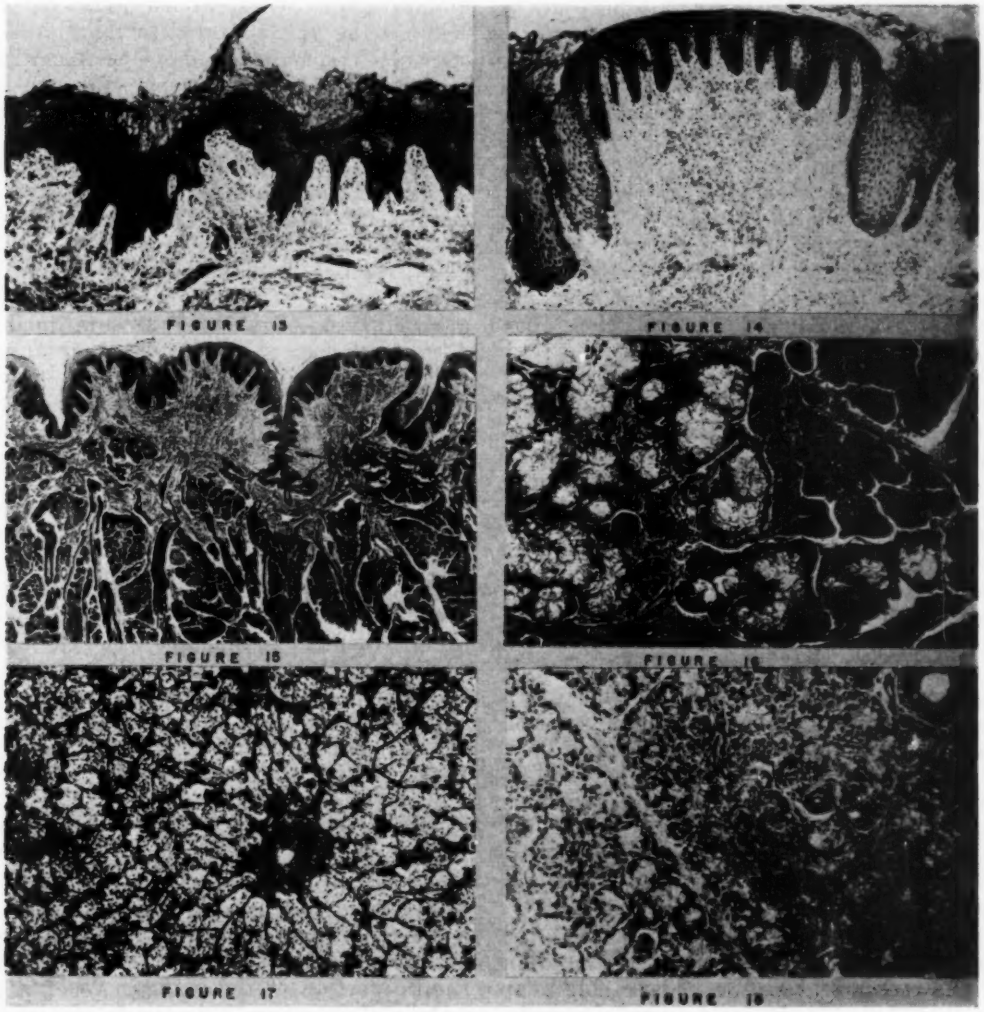


Fig. 13—Filiform papillae with projections of many layers of epithelium and no connective tissue core. x 55.

Fig. 14—A fungiform papilla with true connective tissue core. They do not project above tongue surface. x 55.

Fig. 15—Foliate papillae. Located on lateral aspect of tongue near its base. They possess a few taste buds (t.b.). x 19.

Fig. 16—Lingual glands. Three types of acini—serous, mucous, and mucous with serous demilunes. Hematoxylin and eosin. x 102.

Fig. 17—Parotid gland. One salivary duct lumen shows. Gland composed of purely serios acini. Hematoxylin and eosin. x 230.

Fig. 18—Submaxillary gland. Mucous acini with serous demilunes predominate in this gland. Amount of connective tissue greater than in parotid gland. Hematoxylin and eosin. x 102.

which together completely surround the tube (fig. 22). Upon the appearance of the muscularis mucosae the glands begin to decrease in number and in the lower one third of the tube they are entirely absent (fig. 24). The glands are in the submucosa and penetrate the muscularis mucosae to appear at times in the tunica propria.

The tunica muscularis of the esophagus, although composed of inner and outer layers, is arranged in spiral formations. In sections studied, areas of definitely oblique muscle fibers can be readily demonstrated. The tunica muscularis in the upper half of the esophagus is entirely composed of striated fibers. Near the middle of the tube, smooth fibers appear intermingled with the striated fibers of both layers (fig. 23) and, at the cardiac extremity, the inner layer is composed entirely of smooth fibers while the bulk of the outer layer is made up of nonstriated muscle in which a few striated fibers can be demonstrated (fig. 24).

The Stomach.—Of the four distinct zones in the porcine stomach, the esophageal portion is the smallest. A 60- to 90-cell layer of stratified squamous epithelium lines the esophageal portion. There is a very abrupt change from this stratified squamous epithelium of the esophageal region to the simple columnar epithelium of the cardiac region (fig. 25). In the tunica propria of the esophageal region may be seen a few cardiac glands, appearing only at the immediate junction of the esophageal and cardiac zones. Apparently these glands are the cut ends of true cardiac glands, because at no point can their ducts be traced to the stratified squamous epithelium.

Simple columnar epithelium lines the entire stomach mucosa with the exception of the esophageal region. These columnar lining cells stain similarly to mucous cells, but they are not goblet in shape. The branched tubuloacinar cardiac glands occupy a larger area in the stomach mucosa than either the fundic or pyloric glands. The gastric crypts, which are approximately one fifth of the entire length of the cardiac gland tubes, are lined by simple columnar epithelium. The cells in the neck and body portions of the cardiac glands react to mucous stains, but the cells in the fundic portion of these glands stain similarly to the chief cells of the fundic gland region. When stained with mucicarmine, the cytoplasm of these cells appears granular and does not give the characteristic red mucous stain.

The mucosa of the cardiac diverticulum (fig. 26) is similar to that of the cardiac gland region. In newborn pigs, parietal cells can be demonstrated in the glands of the mucous membrane of the cardiac diverticulum. However, these parietal cells can not be detected in this region in swine 5 weeks of age or over.

There is a gradual transition from the cardiac to the fundic gland regions. The tubules in the fundic gland region appear less branched than those in the cardiac gland region. Tall, columnar cells line the stomach in the fundic gland region. The gastric crypts (fig. 27 and 28) are lined by a cuboidal type of cell. The true fundic gland cells are of two types—chief, or peptic cells, and parietal cells. The columnar cells give a true mucous reaction when stained with mucicarmine.

In the gastric crypts, the cytoplasm of half of the cells facing the lumen of the crypts gives a mucous reaction. In the fundic portion of the fundic glands are cells similar to the mucous cells in the gastric crypts of the fundic gland region, although they differ in shape. The chief and parietal cells do not react to mucous stains. Chief cells stain poorly with hematoxylin and eosin stain, but the parietal cells take on a decidedly pink granular coloration. In the neck of the fundic glands, the parietal cells appear to surround the tube to the exclusion of most of the chief cells. In the body of the gland the chief cells assume the position nearest the lumen and the parietal cells form a complete band around them in a collarlike arrangement. The chief cells and mucous cells are in the majority in the fundic portion of the glands. When a parietal cell does appear in the fundus of the gland, it lies outside the chief cells or mucous cells lining the tube, although at times the cytoplasm of these cells projects toward the lumen between the chief or mucous cells.

There is a gradual transition from the fundic gland region to the pyloric gland region. The tubular pyloric glands do not branch to any considerable extent (fig. 29). The pyloric glands are lined by simple columnar epithelium, which gives a mucous stain when mucicarmine is em-

ployed. The cytoplasm of the pyloric gland cells reacts to stains similarly to the cells in the neck portion of the cardiac glands. The tunica propria is similar throughout the entire stomach. However, more lymphoid tissue is found in the tunica propria of the cardiac region than in the other regions of the stomach. Also,

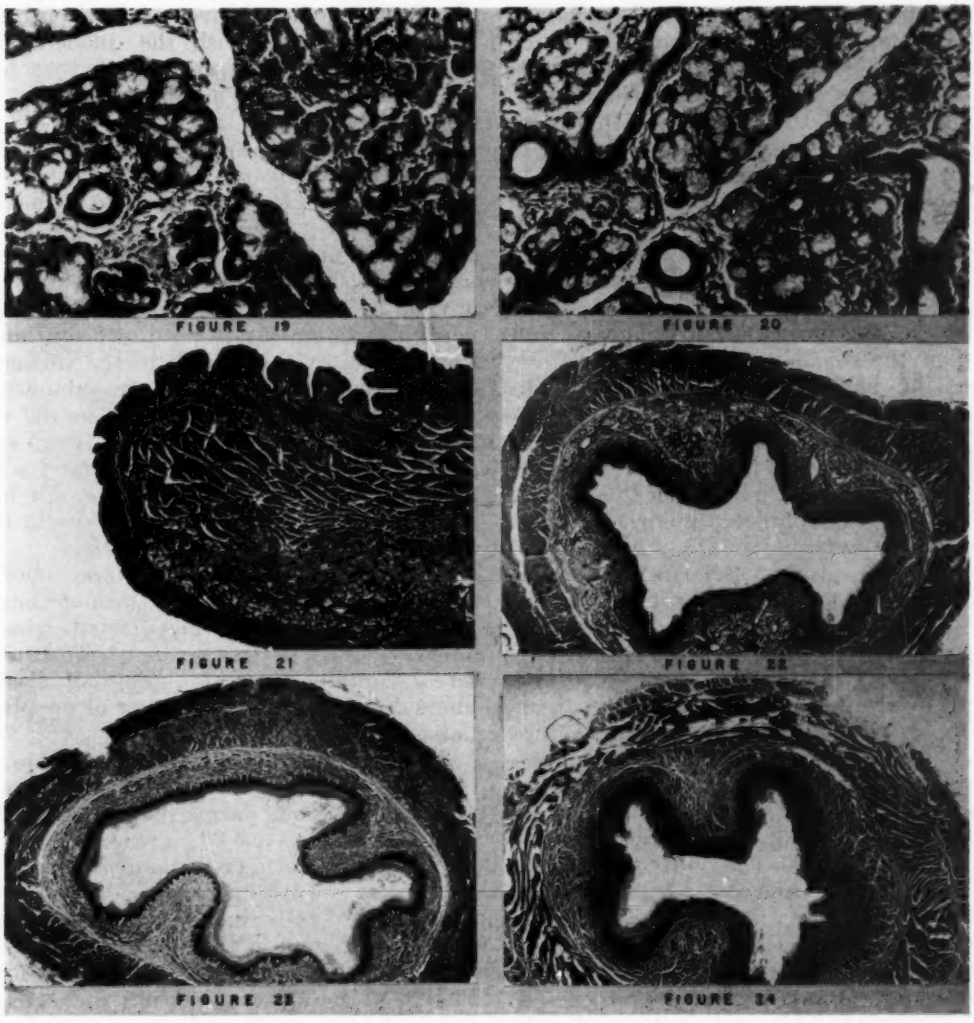


Fig. 19—Sublingual gland, superior part. Predominance of serous acini. Hematoxylin and eosin. x 102.

Fig. 20—Sublingual gland, inferior part. Acini almost entirely mucous; small serous demilunes are demonstrable. Hematoxylin and eosin. x 102.

Fig. 21—Fold between the pharyngeal pouch (above) and esophagus. On the esophageal aspect there are more glands (e.g.) and the depth of the epithelial layer is greater than on the pouch side. x 7.5.

Fig. 22—Oral portion of the esophagus showing complete layer of glands (e.g.) and absence of muscularis mucosae. The tunica muscularis is all striated and cut at various angles, bearing out the fact that these muscles are spiral in direction. x 5.

Fig. 23—Middle esophagus showing the decrease in the number of glands. Muscularis mucosae present in two distinct layers. Striated and nonstriated fibers in tunica muscularis. x 5.

Fig. 24—Cardiac esophagus with no glands present and muscularis mucosae more prominent. Fibers of tunica muscularis chiefly nonstriated. x 5.

there is an increase, over the other parts, in elastic fibers in the tunica propria of the pyloric region (fig. 30).

There is a definite muscularis mucosae in all parts of the stomach. The fibers run in several different directions, in the same region, but an inner circular and an outer longitudinal layer of nonstriated muscle can usually be distinguished. The muscularis mucosae of the pyloric region has an exceedingly large amount of elastic fibers interspersed in it. The submucosa of the stomach is composed of reticular and white fibrous connective tissues, a few elastic fibers, and lymphoid tissue. Embedded in the submucosa are numerous blood vessels, lymph vessels, and nerve ganglia.

The tunica muscularis, in nearly all parts of the stomach, is composed of three layers of nonstriated muscle fibers. In parts of the same section, an inner circular, a middle oblique, and an outer longitudinal layer may be readily demonstrated. The inner and middle layers of fibers form the bulk of the sphincter pylori, although some of the outer layer fibers extend into the sphincter. In the pylorus, the muscle fibers which extend into the torus pyloricus (fig. 31), a prominence extending into the lumen of the pylorus, have no definite arrangement. The muscle fibers are separated by white fibrous connective tissue and numerous elastic fibers. The tunica serosa is composed of white fibrous connective tissue, containing a great number of large elastic fibers. A single layer of mesothelial cells covers the connective tissue.

The Duodenum.—The villi of the duodenum (fig. 32) are covered by simple columnar epithelium, in which numerous large goblet cells occur. The tunica propria is composed of areolar connective tissue, containing lymphocytes, blood vessels, nerves, lacteals, and intestinal glands. As animals become older, there is an increase in the amount of lymphoid tissue in the tunica propria. The cells of the intestinal glands are columnar in type. Along the entire gland duct, goblet cells are seen. The fundic portion of the gland contains cells whose cytoplasm appears intensely pink and granular, when stained with acid dyes. These cells are termed "granular cells of Paneth."

Deeply to the tunica propria is the muscularis mucosae, comprised of a very narrow inner circular and a narrow outer longitudinal layer of nonstriated muscle fibers. The muscularis mucosae is lacking at the junction of the pylorus of the stomach and the duodenum, where it changes from a position deeply to the pyloric glands of the tunica propria to a position in the duodenum, with the duodenal or Brunner's glands deeply to it. A very few disconnected smooth muscle fibers can be observed in the gap.

For the first few centimeters of the duodenum the Brunner's glands form, in the submucosa, a complete layer around the tube (fig. 33). The glands then gradually decrease in numbers and fade out, none being found 53 cm. beyond the pylorus in any of the sections observed. The Brunner's glands appear to be direct continuations of the pyloric glands of the stomach, except that they occur in the submucosa while the pyloric glands occur in the tunica propria. The pyloric and Brunner's glands react similarly, when stained with mucicarmine. They stain less intensely but more granular than the goblet cells in the lining of the stomach and intestines.

The tunica muscularis is composed of a thick inner circular and a thinner outer longitudinal layer of nonstriated muscle fibers. The connective tissue of the tunica serosa contains a great number of elastic fibers deeply to the single layer of mesothelial cells.

The Jejunum.—The jejunal mucosa is lined by simple columnar epithelium, containing numerous large distended goblet cells. The villi (fig. 34) are considerably longer and more narrow than those of the duodenum. The intestinal glands have more goblet cells in the fundic portion than are present in the duodenum. There is an increased number of granular cells of Paneth in the intestinal glands in comparison with the number in the duodenum. Small follicles of lymphoid tissue are observed in the tunica propria. Isolated nodules are seen scattered at irregular intervals along the tube. The muscularis mucosae is thinner than in the duodenum. No glands are present in the submucosa.

The plicae circulares appear higher, narrower, and more numerous in the jejunum than in either the duodenum or ileum. The tunica muscularis is similar in arrangement and structure to that in the duodenum, although the muscle wall as a whole is thinner. The serosa is composed

of white fibrous connective tissue, adipose tissue and beneath the serous lining there is a heavy band of elastic fibers.

The Ileum.—The ileal mucosa is lined by simple columnar epithelium, containing numerous large goblet cells. The villi are shorter than those of the jejunum. The plicae circulares are fewer, shorter, and broader than those of the jejunum. In the fundic portion of the intestinal glands, there are more goblet cells but fewer "cells of Paneth" than in the corresponding zone

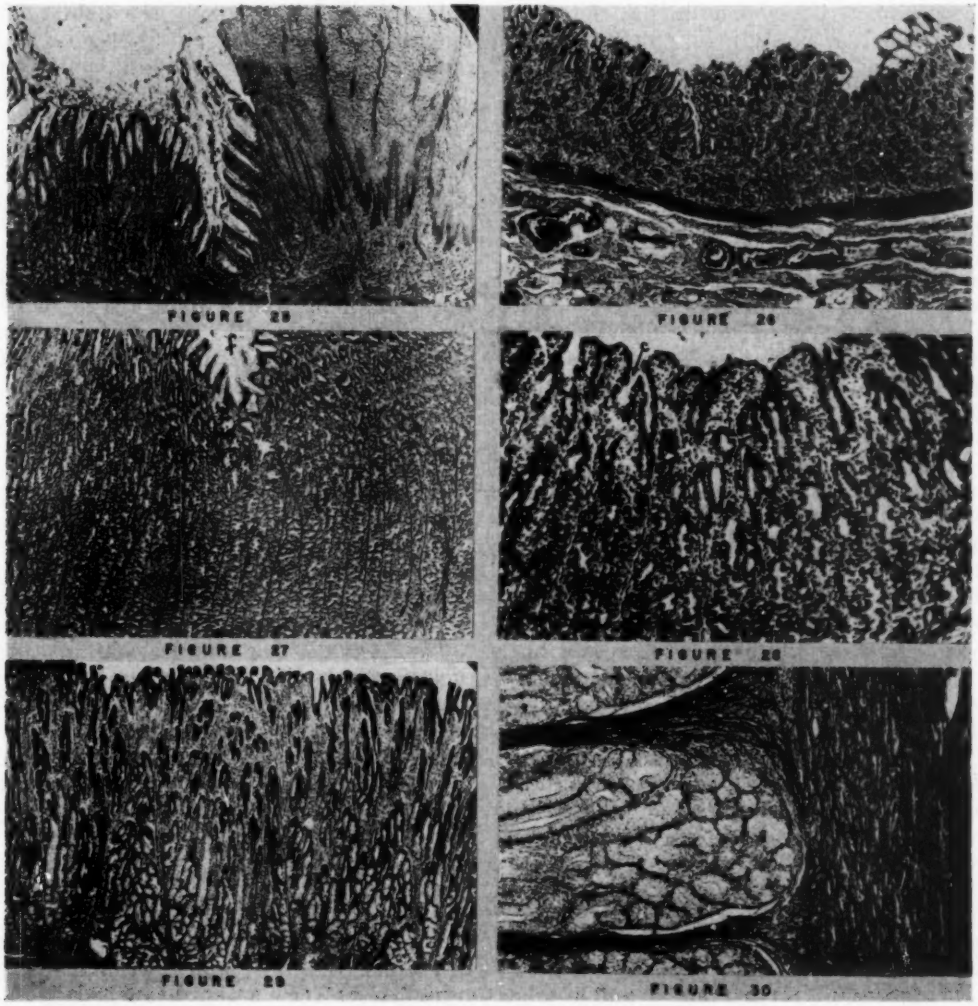


Fig. 25—The abrupt junction of esophageal (right) and cardiac stomach mucosae. Glandless esophageal region lined by stratified squamous epithelium. Glandular cardiac region lined by simple columnar epithelium. x 20.

Fig. 26—Mucosa of cardiac diverticulum. Similar to cardiac region except the glands are shorter. x 20.

Fig. 27—Fundic mucosa. The gastric crypts open into foveolae (f). x 20.

Fig. 28—Fundic glands. Mucous cells line the gastric crypts (c). Parietal cells lie near the lumen in the necks of glands but in the body of the glands chief cells line the lumen with parietal cells collarlike around them. Hematoxylin and eosin. x 75.

Fig. 29—Pylorus of stomach. Glands branch less than in cardiac and fundic regions. Gland cells are wholly mucous in type. x 20.

Fig. 30—Pylorus of stomach. Weigert's stain used to show the vast amount of yellow elastic fibers in the tunica propria. x 75.

of the jejunum. The muscularis mucosae is more distinct in the ileum than in the jejunum. The inner circular layer is thinner than the outer longitudinal layer.

The distinguishing characteristic of the ileum is the large patches of lymphoid tissue occurring in nodular form in the submucosa and at times penetrating the muscularis mucosae and invading the tunica propria (fig. 35). The villi covering the lymphoid areas are shorter than those of the adjacent parts of the ileum. Just anterior to the cecum, the nodules occur in large numbers in the submucosa opposite the mesenteric attachment. The tunica muscularis has a thick inner circular layer and a thin outer longitudinal layer of nonstriated muscle. The tunica serosa is composed of white fibrous connective tissue and a narrow strip of elastic fibers similar to that in the jejunum and duodenum.

The Cecum and the Colon.—The cecum and colon are lined by simple columnar epithelium containing numerous large goblet cells. Almost all of the cells of the intestinal glands and mucosa of these viscera are mucous in type. No granular "cells of Paneth" are demonstrable in the glands of either the cecum or colon. The villi end abruptly at the ileocecal junction (fig. 36) and are not noticeable throughout the large intestine. Plicae circulares are present but in fewer numbers than in the small intestine. An inner circular and outer longitudinal layer of nonstriated fibers comprise the muscularis mucosae.

The submucosa of white fibrous connective tissue and elastic fibers contains large amounts of adipose tissue and lymph follicles. The tunica muscularis is composed of an inner circular layer of nonstriated muscle fibers and an outer longitudinal layer, which in places increases in thickness to form the taeniae (fig. 37). In the cecum, there are three taeniae and in the colon only two. There is a considerable increase in the amount of elastic fibers present in the taeniae as compared to the amount found in the remainder of the longitudinal muscle layer. The taeniae of the cecum in the region where the mesentery is adherent is much thinner than the other two taeniae. The walls of the cecum and colon are thrown into bulges forming recesses or haustra because of the tension of the taeniae. The tunica muscularis is carried into the folds between the haustra (fig. 38). In the folds, the inner circular layer of muscle fibers is much thinner than it is in the other parts of the cecum and colon. The tunica serosa is similar to that of the small intestine.

The Rectum.—The mucosa of the rectum is lined by simple columnar epithelial cells, containing relatively few goblet cells. The intestinal glands appear to be separated from each other by less tunica propria (fig. 39) than is the case in the colon. The cells of the rectal glands are mucous in type. The muscularis mucosae is thicker in the rectum than in any other portion of the intestine, being composed of two definite layers; the intestinal glands appear to rest on it in many places.

In the submucosa, there is considerably more lymphoid and adipose tissue than in the colon. The tunica muscularis is composed of an inner circular and an outer longitudinal layer of nonstriated muscle, which contains large numbers of elastic fibers. There are no taeniae in the wall of the rectum, but there is an increase in the relative thickness of the tunica muscularis as compared to that found in the colon. A typical serosa surrounds the anterior portion of the rectum, while the posterior portion, being retroperitoneal, is covered only by a fibrous adventitia.

The Anus.—The simple columnar epithelium of the rectum changes abruptly to stratified squamous epithelium at the anus (fig. 40). Near the exterior of the canal, there is a keratinized layer and deeply to it the many-layered stratified squamous epithelium. Numerous hair follicles are seen in the epithelium near the exterior opening of the tract and should be properly considered a part of the skin. There is a gradual fading out of the muscularis mucosae in the region of the junction of the rectum and anus. In the tunica propria of the anus can be seen a few branched tubular gland-like structures; these are probably cross sections of intestinal glands. Further exteriorly are the nonstriated muscle fibers of the M. sphincter and internus. Beyond this muscle are the striated fibers of the M. sphincter ani externus.

The Gall Bladder.—The gall bladder is lined by a simple columnar epithelium in which numerous goblet cells occur. The epithelium is thrown into tall villi by areolar connective tissue papillae, rich in elastic fibers (fig. 41). No muscularis mucosae

is present in the wall of the gall bladder. The circular layer of the tunica muscularis of nonstriated muscle is interspersed with large amounts of white fibrous connective tissue and elastic fibers. The outer longitudinal layer of the tunica muscularis is not isolated by as much connective tissue as the inner circular layer. The subserous layer is made up chiefly of white fibrous connective tissue, containing a few elastic fibers.

The Common Bile Duct.—The cystic and

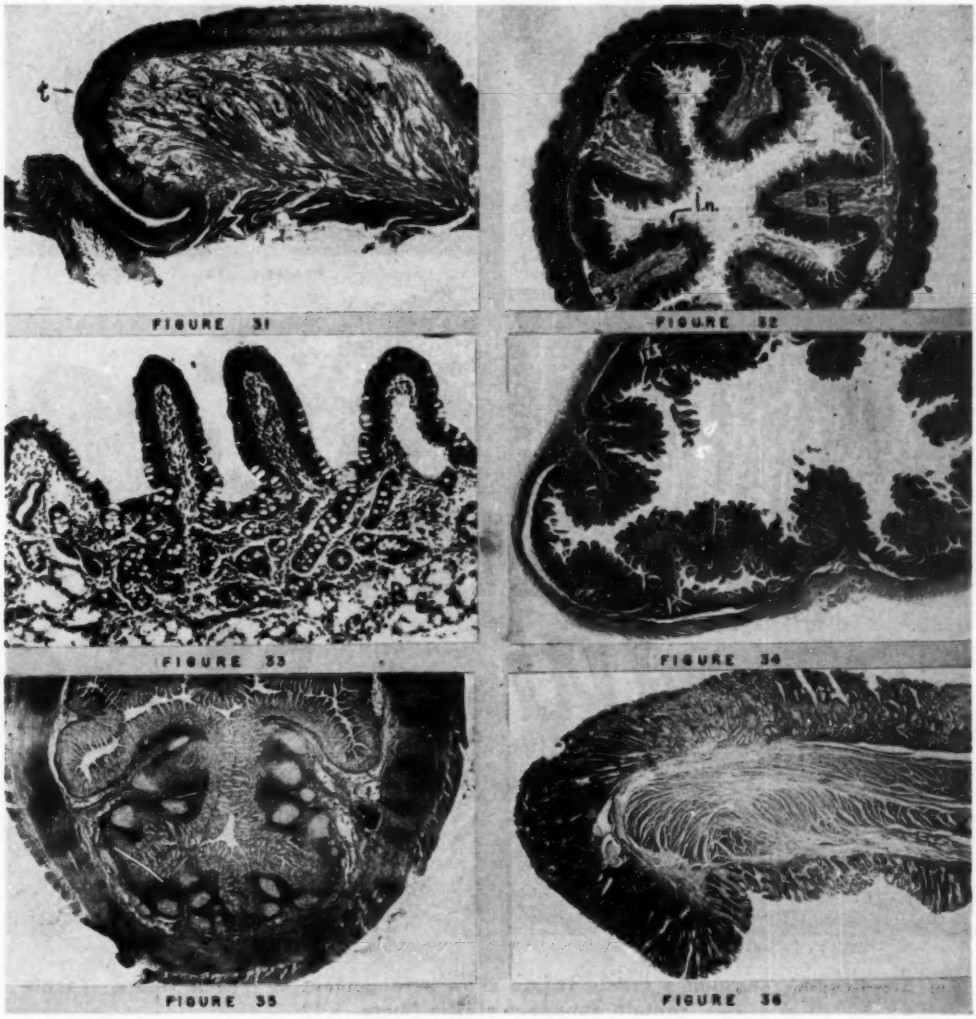


Fig. 31—Pyloric and duodenal (left) mucosae. Circular and oblique muscle fibers make up the torus (t). Few longitudinal fibers are evident. x 2.

Fig. 32—Duodenum showing extensive Brunner's gland (B.g.) beneath a definite muscularis mucosa. Also one solitary lymph nodule (l.n.). x 5.

Fig. 33—Duodenum showing relationship of Brunner's (B.g.) and Lieberkuhn's gland (L.g.) with goblet cells in epithelial lining and Lieberkuhn's glands. A large lacteal is in right villus. x 75.

Fig. 34—Jejunum. The plicae are narrower than in duodenum and ileum and the tunica muscularis is more limited. x 5.

Fig. 35—Ileum showing aggregations of lymph nodules in submucosa pushing muscularis mucosa toward lumen and entering the tunica propria. x 5.

Fig. 36—Border of the ileocecal valve showing the ileal mucosa above, cecal mucosa below. x 7.5.

hepatic ducts join to form the common bile duct. The common bile duct enters the duodenum alone, approximately 1.7 cm. posterior to the torus of the pylorus. The duct is lined by simple columnar epithelium in which large goblet cells are observed (fig. 42). These lining cells are similar to the cells lining the gall bladder,

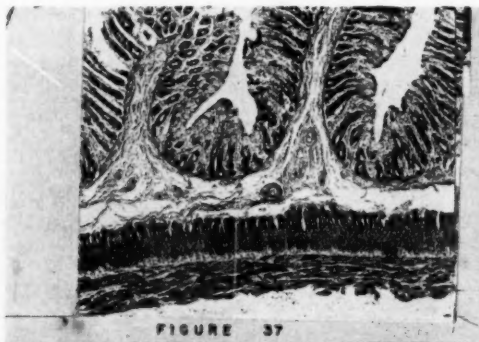


FIGURE 37

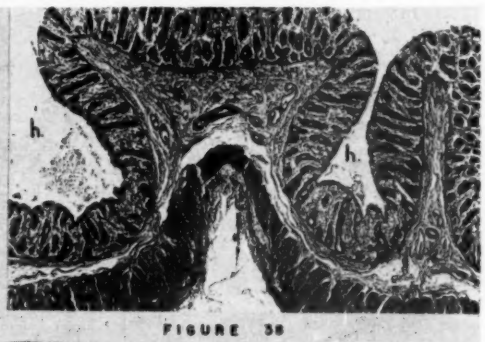


FIGURE 38

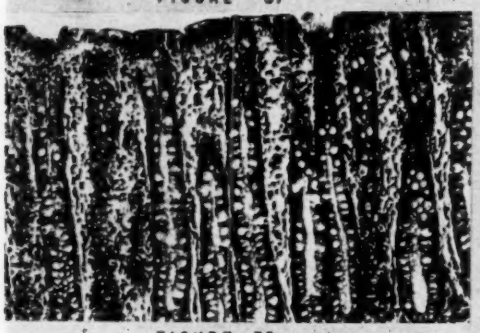


FIGURE 39

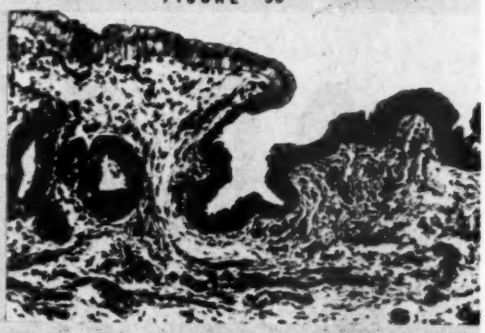


FIGURE 40

FIGURE 41

FIGURE 42

Fig. 37—Cecum. Through a taenia (t.) to show arrangement of muscle fibers. Taenia contain abundant elastic fibers. x 19.

Fig. 38—Cecum. Fold between two haustra (h); circular muscle is thinner in the fold. x 19.

Fig. 39—Rectal mucosa showing few goblet cells in the lining mucosa but distended mucous cells in the glands. x 82.

Fig. 40—Junction of rectum (left) and anus. Simple columnar epithelial cells change abruptly to stratified squamous and the muscularis mucosae fades out completely. x 110.

Fig. 41—Gall bladder. The lining cells are mucous in type. No muscularis mucosae but muscle fibers are scattered through corium. Subserous layer contains numerous yellow elastic fibers. x 32.

Fig. 42—Duodenal (above) and pancreatic duct mucosae. The mucous cells of the pancreatic duct are larger than the goblet cells of the duodenum. The two sets of glands are separated by connective tissue (c.t.) only. x 50.

both in structure and staining properties. Numerous branched tubular glands are scattered throughout the tunica propria of the duct. The cells of these glands give a definite mucous reaction when they are stained with mucicarmine. The tunica muscularis is comprised of two layers of nonstriated muscle fibers. These layers are not as definite as they are in the gall bladder. The inner circular layer has less connective tissue separating the muscle fibers than the corresponding layer in the gall bladder. The outer longitudinal layer is similar to that of the gall bladder.

The Pancreatic Duct.—The pancreatic duct opens into the duodenum 10 to 13 cm. posterior to the opening of the bile duct. It enters obliquely from anterior to posterior and is difficult to locate macroscopically as it does not open through a papilla. The duct is lined by tall, simple, columnar epithelium composed almost entirely of distended goblet cells which are exceptionally large (fig. 42). These cells are continuous with the columnar epithelial cells lining the duodenum, at the point at which the duct opens into its lumen. The portion of the tunica propria deeply to the epithelium is composed of areolar connective tissue, containing numerous lymphocytes. The remainder of the tunica propria, composed of white fibrous connective tissue and elastic fibers, contains numerous branched tubular glands. The cells of these glands react to Delafield's hematoxylin, similarly to the goblet cells lining the pancreatic duct.

After the pancreatic duct has pierced the tunica muscularis of the duodenum, it continues posteriorly for some distance in the submucosae before piercing the mucous membrane. White fibrous connective tissue separates the glands of the duodenum from the glands of the pancreatic duct. The pancreatic duct has neither a muscularis mucosae or a tunica muscularis. The circular layer of the tunica muscularis lying exterior to the duct appears thickened where it crosses over the duct. This thickening band of the muscle thus runs parallel with the duct in its course through the submucosa.

CONCLUSIONS AND SUMMARY

The entire digestive tract of *Sus scrofa domestica* is composed of a mucous membrane, a corium, and a tunica muscularis. The muscularis mucosae first appears in the esophagus, in the region opposite the cricoid cartilage, and is then present to the anus, except for a very short distance in the region of the junction of the pylorus of the stomach and duodenum.

The labial glands are poorly developed. They can be found farther from the commissure in the lower lip than in the upper lip.

Tactile cells are very prominent in the porcine hard palate.

In the midregion of the aboral surface of the soft palate, there is a gradual transition from stratified squamous to pseudostratified ciliated columnar epithelium.

Taste buds are found on the fungiform, foliate, and circumvallate papillae of the tongue. The most buds are to be found on the circumvallate papillae.

The parotid gland is purely serous in type. The submaxillary, sublingual, buccal, labial, and some of the lingual glands are mixed in type.

The pharynx is definitely divided into an oral portion lined with stratified squamous epithelium and a nasal portion lined with columnar epithelium.

The wall of the pharyngeal pouch is similar in structure to that of the pharynx.

Esophageal glands form a complete layer around the esophagus in its initial portion, gradually thinning out, until in the posterior third of the tube they are entirely absent. The muscularis mucosae is lacking in the initial portion of the esophagus. The tunica muscularis of the esophagus can not be demonstrated as a complete inner circular and outer longitudinal layer of muscle fibers but the two layers are present in a slightly spiral arrangement. In the oral portion of the esophagus, only striated fibers occur; in the midportion, striated and nonstriated fibers are seen; and in the cardiac portion, only a few striated fibers intermingle with the nonstriated fibers.

Variations in the structure of the stomach mucosae divide it into esophageal, cardiac, cardiac diverticulum, fundic, and pyloric regions. The esophageal portion is nonglandular. The cardiac and cardiac-diverticulum regions are similar in structure except in the newborn pigs when parietal cells are found in the cardiac diverticulum but not in the cardiac glands. Mucicarmin stain reveals mucin in the

cells in the neck and body portions of the cardiac glands, the cells lining the gastric crypts of the fundic glands, the mucous cells in the fundic portion of the fundic glands, and in the pyloric gland cells. The cells in the fundic portion of the cardiac glands and the chief cells of the fundic glands appear to be similar. The parietal cells of the fundic glands form a collarlike arrangement around the chief cells in the body of the glands. The mucous and chief cells are in the majority in the fundus of the fundic gland region. The few parietal cells in the fundus of the fundic glands lie to the exterior of the chief cells or mucous cells, although their cytoplasm at times points toward the lumen.

The pyloric glands are continuous with Brunner's glands, except the muscularis mucosae lies beneath the former and is superficial to the latter. For several centimeters of the duodenum, Brunner's glands form a complete layer around the tube and then gradually decrease. They were not observed beyond 53 cm. from the pylorus.

The jejunum contains more "cells of Paneth" in its glands than does the duodenum. No glands are present in the submucosa of the jejunum.

The outstanding characteristic of the ileum is the large lymph nodules in the submucosa, which push the muscularis mucosae lumenward and penetrate the tunica propria.

The cecal mucosa contains no villi and no granular "cells of Paneth" are demonstrable in its glands. Three taeniae are seen in the tunica muscularis of the cecum.

The colon is similar to the cecum in structure except that the tunica muscularis has only two taeniae.

The lining cells of the rectum are similar to those in the cecum and colon. There are no taeniae in the tunica muscularis of the rectum, but there is an increase in the amount of elastic fibers present.

The anus is lined by stratified squamous epithelium and has no muscularis mucosae. In the corium of the anus are a few branched tubular, glandlike structures.

The gall bladder wall has no muscularis mucosae and no glands in the corium. The bile duct enters the duodenum alone approximately 1.7 cm. posterior to the torus of the stomach. Branched tubular glands occur in the submucosa of the bile duct but the muscularis mucosae is lacking.

The pancreatic duct passes posteriorly and obliquely through the wall of the duodenum 10 to 13 cm. posterior to the bile duct opening. The pancreatic duct does not have a muscularis mucosae or a tunica muscularis. In the region where the pancreatic duct pierces the tunica muscularis of the duodenum, there is a definite thickening in the inner circular layer of the muscle, indicating that perhaps this acts as a valve.

Bibliography

Bärner, M.: Über die Backendrüsen der Haussäugetiere. Arch. f. wissensch. u. prakt. Tierheilk., *19*, (1893): 149-179.

Barthol, A.: Beiträge zur Anatomie und Histologie der Magenschleimhaut von Sus Scrofa Domestica-Inaug. Dissert. Buchdruckerei Otto Franke, Dresden (1914): 1-57.

Baumgartner, W. J.: A Laboratory Manual of the Foetal Pig. The Macmillan Co., New York, N. Y. (1924): 1-57.

Bensley, R. R.: The Differentiation of the Specific Elements of the Gastric Glands of the Pig. Am. J. Anat., *2*, (1903): 105-156.

Bensley, R. R.: The Structure of the Glands of Brunner. Univ. of Chicago Press, Chicago, *10*, (1903): 279-326 .

Bensley, R. R.: The Cardiac Glands of the Mammalian Stomach. Anat. Rec., *4*, (1910): 375-390.

Brade, G. Von: Zur Histologie des Magens des Schweines. Bericht über das Veterinärwesen im Königreiche Sachsen für das Jahr 1882. G. Schönfeld's Verlagsbuchhandlung, Dresden (1883): 132-140.

Corner, G. W.: The Structural Unit and Growth of the Pancreas of the Pig. Am. J. Anat., *16*, (1914): 207-236.

Dettweiler, Fr., und Müller, K.: Lehrbuch der Schweinezucht. Verlagsbuchhandlung Paul Parey, Berlin (1924): 81-94.

DuBois, C. C.: Granule Cells in the Mucosa of the Pig's Intestine. Anat. Anz., *25*, (1904): 6-16.

Edelmann, R.: Vergleichende anatomische und physiologische Untersuchungen über eine besondere Region der Magenschleimhaut (Cardiadrüsenregion) bei den Saügethieren. Deutsche Ztschr. f. Thiermed., *15*, (1889): 165-214.

Ellenberger, W., und Baum, H.: Handbuch der vergleichenden Anatomie der Haustiere. Sechzehnte Auflage. Verlag von Julius Springer, Berlin (1926): 354-457.

Ellenberger, W., und Hofmeister, V.: Der Magensaft und die Ristologie der Schweine. Arch. f. wissensch. prakt. Tierh., *11*, (1885): 249-268.

Ellenberger, W., und Schumacher, S.: Von d. Histologie der Haussäugetiere. Vierte Auflage. Verlagsbuchhandlung Paul Parey, Berlin (1914): 188.

Ellenberger, W., und Trautmann, A.: Histologie der Haussäugetiere. Fünfte Auflage, Verlagsbuchhandlung Paul Parey, Berlin (1921): 110-178.

Flint, J. M.: The Angiology, Angiogenesis, and Organogenesis of the Submaxillary Gland. Am. J. Anat., *2*, (1902-1903): 415-444.

Flint, J. M.: The Organogenesis of the Oesophagus. Anat. Anz., *30*, (1907): 442-451.

Fröbisch, T. A.: Beiträge zur vergleichenden Histologie des Gaumensegels der Haussäugetiere. Inaug. Dissert., Buchdrucherei Otto Franke, Dresden, 1912.

Fröhlich, A.: Untersuchungen über die Uebergangszenen und einige Eighenthümlichkeiten des feineren Baues der Magenschleimhaut der Haussäugetiere. Inaug. Dissert., Leipzig (1907): 1-136.

Goetsch, Emil: The Structure of the Mammalian Oesophagus. Am. J. Anat., *10*, (1910): 1-40.

Grahame, T.: Structures of the Oesophagus of Domestic Animals. Vet. Rec., n.s. *6*, (1926): 308-311.

Greenwood, Marian: Observations of the Gastric Glands of the Pig. J. Physiol., *5*, (1884-1885): 195-208.

Haane, Gunnar: Über die Cardiadrüsen und die Cardiadrüsenzone des Magens der Haussäugethiere. Arch. f. Anat. u. Physiol., Anatomische Abt. (1905): 1-32.

Hering, Ewald: The Liver (In Salomon Stricker's, Human and Comparative Histology). The New Sydenham Society, London, *2*, (1872): 1-33.

Hock, J.: Untersuchungen über den Uebergang der Magen in die Darmschleimhaut mit besonderer Berücksichtigung der Lieberkuhn'schen Krypten und Brunner'schen Drüsen bei den Haussäugetieren. Inaug. Dissert., Giessen (1899): 1-80.

Houpert, Louis: Recherches sur la musculature de la langue. Arch. d'anat., d'histol., et d'embryol., *8*, (1928): 5-79.

Hunter, R. H.: A Contribution to the Development of the Duodenum. J. Anat., *61*, (1926): 206-212.

Illing, George: Verleichende Histologische Untersuchungen über die Leber der Haussäugetiere. Anatomische Abt., *26*, (1905): 184-190.

Illing, George: Verleichende Makroskopische und Mikroskopische Untersuchungen über die Submaxillaren Speicheldrüsen der Haussäugetiere (Aus dem Physiolog. und Histolog. Institut. der Tierärztlichen Hochschule zu Dresden). Anat. Hefte, *26*, (1904): 387-526, Abt. 1.

Killian, Gustav: Über die Bursa und Tonsilla pharyngea. Morphologisches Jahrbuch, *14*, (1888): 660-662, 684-685.

Kirk, E. G.: On the Histogenesis of Gastric Glands. Am. J. Anat., *10*, (1910): 473-520.

Kunze, Oscar: Zur vergleichenden mikroskopischen Anatomie der Organe de Maulhöhe, des Schlundkopfes und des Schlundes der Haussäugethiere. Deutsche Zeitschrift für Thiermedicin und Vergleichende Pathologie, Elfter Band (1885): 1-44.

Lewis, F. T., and Bremer, J. L.: Textbook of Histology. P. Blakiston's Son and Co., Philadelphia, 1927.

Lim, Robert K. S.: The Gastric Mucosa. Quart. J. Micros. Sci., *66*, (1922): 187-212.

McGill, Caroline: The Structure of Smooth Muscle in the Resting and in the Contracted Condition. Am. J. Anat., *9*, (1909): 439-545.

McGill, Caroline: Histogenesis of Striated Muscle. Anat. Record, *4*, (1910): 23-47.

Mönning, G.: Zur Histologie der Cardiadrüsen von Sus scrofa. Inaug. Dissert., Zürich, Dresden (1907): 1-67.

Montane, L., and Bourdelle, E.: Anatomie régionale des animaux domestiques. III. Porc. Librairie J.-B. Baillière et Fils, 19 Rue Hautefeuille, Paris (1919): 24-31.

Oppel, A.: Lehrbuch d. vergleichenden mikroskopischen Anatomie—1. des Magens. G. Fischer, Jena (1896): 245-256.

Oppel, A.: Verdauungsapparat. Ergebn. d. Anat. u. Entwicklungsgesch, *7*, (1898): 29-102.

Ross, Mary J.: The origin and Development of the Gastric Glands of Desmognathus, Amblystoma and Pig. Biol. Bull., *4*, (1902): 66-95.

Tang, E. H.: Über die Panethschen Zellen sowie die gelben Zellen des Duodenums beim Schwein und den anderen Wirbeltieren. Arch. f. Mikr. Anat. Bd., *96*, (1922): 182-209.

Sobotta, J.: Atlas of Human Histology and Microscopic Anatomy. Vol. I & II. Trans. by W. H. Piersol, G. E. Stechert & Co., N. Y., 1930.

Wlassow, K.: Zur Entwicklung des Pankreas beim Schwein. Morphol. Arb., (Aus. d. Anat. Inst. zu Freiburg i.b.), *4*, (1895): 67-77.

Laboratory Studies on Erysipelas
I. Immunization Against Swine Erysipelas and Susceptibility of Swine to Challenge

M. S. COOPER, Ph.D.; G. PERSONEUS, B.S.; M. J. HARVEY, D.V.M.; R. C. PERCIVAL, V.M.D.

Pearl River, New York

In a previous paper [1] the history of vaccination against *Erysipelothrix rhusiopathiae* infection was reviewed. The preparation and testing of a bacterin * similar to those developed by Traub [2] and Dinter [3] was discussed. Studies in turkey poults were reported which indicate that vaccinated animals were protected against a severe laboratory challenge.

In this paper, we shall report on the experimental immunization of swine with this bacterin as tested by the scarification procedure of Fortner and Dinter,[4] and some observations on attempts at enhancing *Ery. rhusiopathiae* infection in swine.

Immunization of Swine with an Adsorbed Erysipelothrix Rhusiopathiae Bacterin

Materials and Methods

Bacterin.—The preparation of the bacterin was described in the previous communication.[1] The several lots used in these experiments were tested in mice and found to have a $p.d._{50}$** of at least 1:150.

For immunization purposes, 1 dose of 5 cc. was administered subcutaneously into the axilla.

Challenge Cultures.—Three cultures of *Ery. rhusiopathiae,*† 846, 847, 848, were cultivated at 37 C. for eighteen hours in yeast bile broth containing 10 per cent horse serum.

Experimental Animals.—Forty-seven apparently normal pigs, of the Yorkshire type (mixed breed) obtained in Maryland, were used for these studies. Their weight at vaccination was 40 to 60 lb.

Experimental Design.—Two experiments were planned, one carried out after the completion of the other. The pigs used in both experiments represented eight litters. The plan of each experiment is shown in table 1.

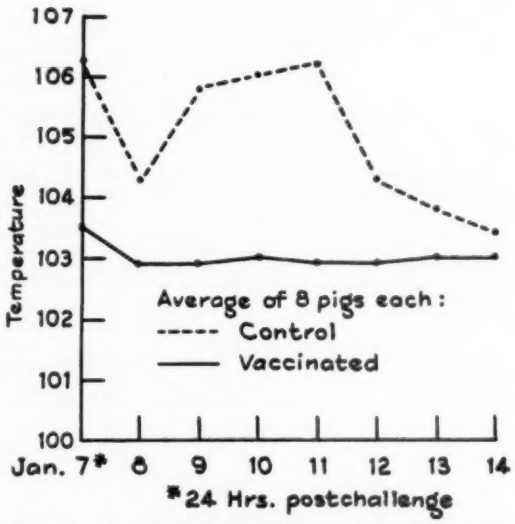


Fig. 1—Average temperature curves, experiment A.

Experiment A

The 16 pigs used were divided into two equal groups, one for vaccination and one for unvaccinated controls. One pig from each of the eight litters was placed in each group.

Experiment B

The 31 pigs used were divided into five groups. One pig from each litter comprised a control group of 8. The remaining pigs were distributed at random into four groups of 5, 6, and 7 pigs; each group was to be vaccinated with one of four lots of bacterin.

Challenge

Approximately three weeks after administration of the single dose of bacterin, both vaccinated and control pigs were challenged by the following procedure. One side only of each pig was trimmed with an electric clipper. At regular intervals, four vertical scarifications were made with the tip of a scalpel which had been bent and roughened. The scarifications were just deep enough to produce a slight serum ooze tinged with blood. In no case was persistent bleeding observed. Each of the three cultures of *Ery. rhusiopathiae* was placed on each

From the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The authors are indebted to Mr. F. Katt and his assistants for their invaluable aid in performing the swine studies, and to Mrs. D. Budd for assistance in preparation of the manuscript.

* Duovax® Erysipelas Bacterin, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

** The dose which protects 50 per cent of the animals tested.

† Obtained from Dr. K. L. Wolters, Germany, where they are used for swine scarification by the Fortner procedure.

of three scarifications separately, using a pullorum dropper. Each scarification was rubbed vigorously with a glass rod. The rearmost scarification was left untouched as a control.

RESULTS

Experiment A.—Seven of 8 control pigs showed marked swelling and erythema at and around scarification sites within twenty-four hours. All control pigs showed a definite rise in temperature (fig. 1). The swelling and erythema spread to a maximum of about 3 in., covering the scarification line during the third and fourth days postchallenge and then began to subside.

All vaccinated pigs showed only minimal swelling at the scarification sites. Temperatures remained well within normal limits through the postchallenge observation period (fig. 1).

Experiment B.—All control pigs reacted as described in experiment A. Likewise, vaccinated swine exhibited only a minimal reaction at the scarification sites which completely cleared in forty-eight hours. Temperature reactions in all pigs were similar to those in experiment A, as shown in figure 2.

ATTEMPTS AT ENHANCING ERYSIPELOTHRIX RHUSIOPATHIAE INFECTION IN SWINE

Although the scarification procedure of Fortner has been used successfully by several workers outside of Germany, such as Shuman,[5] Nardelli,[6] and Bakos,[7] others have obtained rather inconsistent results. Basset[8] has found groups of swine in which about 30 per cent were completely refractory to this challenge procedure.

EXPERIMENT C—EFFECT OF "PREPARATION" BY PRIOR VACCINATION ON SUSCEPTIBILITY OF PIGS

Doyle,[9] and Thomson and Gledhill[10] also reported inconsistencies in the scarification procedure and devised a method of "preparing" pigs by prior vaccination against hog cholera. In their studies, pigs were vaccinated with crystal violet hog cholera vaccine and challenged three weeks later with virulent hog cholera virus. After an additional

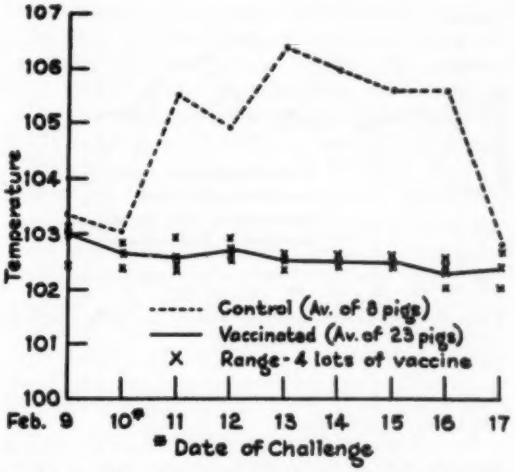


Fig. 2—Average temperature curves, experiment B.

three weeks, a portion of the hog cholera-vaccinated and challenged pigs were vaccinated against swine erysipelas, with the remainder left as controls. After a lapse of either four or ten weeks, erysipelas-vaccinated and control pigs were challenged intramuscularly with 2 ml. of a six-hour growth of *Ery. rhusiopathiae.* None of the erysipelas-vaccinated pigs showed local or general symptoms of erysipelas. The control pigs, however, reacted "violently." Three were killed *in extremis* two and five days after challenge; 2 were severely affected but eventually recovered.

The following is a report on our experience with a similar method.

MATERIALS AND METHODS

Vaccine.—Crystal violet vaccine (Fort Dodge) was injected intramuscularly in 5-cc. amounts.

TABLE 1—Results Following Challenge by Scarification of Pigs Vaccinated with Adsorbed Erysipelothrix Rhusiopathiae Bacterin and Unvaccinated Controls

No. of pigs	Lot of bacterin	Date		Results	
		Vaccinated	Challenged	Elev. Temp.	Swelling and erythema
			Experiment A		
8	26	12/11/52	1/6/53	0/8	8/8 minimal
8	....	Controls	1/6/53	8/8	7/8 marked; began to subside 3rd and 4th day
			Experiment B		
5	56	1/16/53	2/10/53	0/5	5/5 minimal; completely cleared in 48 hr.
7*	26	1/16/53	2/10/53	0/7	7/7 minimal; completely cleared in 48 hr.
5	62	1/16/53	2/10/53	0/5	5/5 minimal; completely cleared in 48 hr.
6	53	1/16/53	2/10/53	0/6	6/6 minimal; completely cleared in 48 hr.
8	....	Controls	2/10/53	8/8	8/8 marked; began to subside 3rd and 4th day.

* Two pigs were inadvertently vaccinated with a 10-cc. dose. No reactions were noted after injection.

Challenge Materials.—A 10^{-3} dilution of virulent hog cholera virus was used and three cultures of *Ery. rhusiopathiae*, 846, 847, 848, as in the bacterin experiment.

Experimental Animals.—Ten Yorkshire pigs, weighing 60 to 75 lb., were obtained in Canada for use in this experiment.

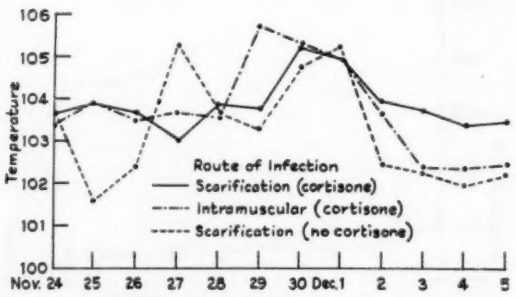


Fig. 3 — Average temperature curves, experiment D.

Procedure.—Five of the 10 pigs were vaccinated with 5 cc. of crystal violet vaccine on July 9, 1953; the other 5 were left unvaccinated. On Aug. 6, 1953, each of the 5 vaccinated animals was challenged with 1 ml. of 10^{-3} dilution of virulent hog cholera virus injected intramuscularly. These swine were observed closely for 21 days postchallenge. During this observation period, all 5 challenged animals exhibited pyrexia, inappetence, and symptoms of incipient pneumonia. On the eighth postvaccination day. sulfamethazine was administered, intraperitoneally, at a dosage of 1½ gr. per pound of body weight the first day, and ½ gr. per pound per day for two additional days, after which these pigs were apparently normal.

On August 26, the 5 hog cholera-vaccinated and challenged pigs and the 5 controls were each injected intramuscularly with 7 ml. of a culture of *Ery. rhusiopathiae* consisting of equal proportions of cultures 846, 847, and 848.

Results

Four of the 5 animals previously "prepared" with crystal violet vaccine and virulent virus, and all controls developed hyperthermia following injection with *Ery. rhusiopathiae* cultures. Two of the 5 controls developed an erythema about the injection site. There were no other deviations from normal.

Experiment D—Effect of Cortisone on Susceptibility of Pigs

Since the introduction of cortisone by Hench,[13] numerous reports have been published indicating that cortisone may decrease the resistance of laboratory animals to infection. Berlin[14] indicated that cortisone increases the virulence of normal inhabitants of the gastrointestinal tract in mice. Klingman[15] noted that the severity of *Staphylococcus aureus* infection in guinea pigs was enhanced by cortisone.

The purpose of this study was to determine if cortisone would influence the severity of the response to several methods of infecting swine with *Ery. rhusiopathiae*.

Materials and Methods

Materials.—Cortisone acetate (Merck) was administered intramuscularly and three cultures of *Ery. rhusiopathiae*, 846, 847, 848, as in the bacterin experiment.

Experimental Animals.—Ten pigs, consisting of two litters of 5 pigs each, weighing approximately 30 to 40 lb., were used in this study. These pigs were obtained in Maryland from the same source as described in the bacterin experiment.

Procedure.—The two litters were equally distributed in two groups of 4 each for the administration of cortisone, and one group of 2 was maintained as controls, as shown in table 2. Cortisone was administered intramuscularly in a single daily dose of 5 mg. per pound of body weight, starting two days before infection with *Ery. rhusiopathiae* and continuing for fifteen days.

Each of 4 pigs in one group was injected intramuscularly into the ham with 10 ml. of a mixture of the 3 cultures of *Ery. rhusiopathiae*. Each of the second group of 4, and the 2 controls, was infected by scarification as described previously.

Results

One of the 4 pigs treated with cortisone and infected by the scarification procedure died seven days after infection. *Erysipelo-*

TABLE 2—Effect of Cortisone Administered to Unvaccinated Pigs on Severity of Reaction to Challenge with Erysipelothrix Rhusiopathiae

No. of pigs	Cortisone	Route of infection	Results		
			Swelling and erythema	Elev. Temp.	Other
4	Single-daily dose, 5 mg./lb., i.m., 2 days before infection for 15 days	Intramuscular	4/4 minimal at injection site	Febrile period appeared to be delayed	
4		Scarification	4/4 distinct	Febrile period appeared to be delayed	1 death 7 days postinfection; *Ery. rhusiopathiae* isolated
2	Controls	Scarification	2/2 distinct	2/2	

thrix rhusiopathiae was isolated from all tissues cultured. All other animals survived.

All animals infected by the scarification method showed distinct erythema and swelling at the infection site as described in the bacterin experiment. There were no differences observed in the severity of erythema between the cortisone-treated group and the 2 controls. The 4 animals infected by the intramuscular route showed only a minimal erythema at the injection site. Averages of body temperatures (fig. 3) appeared to indicate that, to some extent, cortisone did delay the febrile period. The characteristic double peak in temperature was absent in cortisone-treated animals, and hyperthermia persisted for a slightly longer period.

DISCUSSION

The value of various methods of immunizing swine against *Ery. rhusiopathiae* can best be determined by laboratory challenge tests. The employment of field trials for information of this sort is extremely valuable, but the results obtained can not replace critical laboratory studies.

The role of *Ery. rhusiopathiae* in the syndrome known as erysipelas of swine has been questioned with a good deal of justification by Doyle,[11] and others, probably due to the inability to produce the classical disease in the laboratory.

In our experiments, the scarification test of Fortner was successfully used. We believe that the lack of skin lesions coupled with the lack of change in body temperature after challenge is a good indication that the animal had been immunized against *Ery. rhusiopathiae* infection.

We have seldom been able to kill a pig by this procedure, and our inability to do this is in close agreement with Shuman's findings. This fact does not rule out the possibility that severe losses under field exposure may be due to an association of *Ery. rhusiopathiae* with other disease conditions, making possible the development of an *Ery. rhusiopathiae* septicemia. The above statement is not merely conjecture and we have already performed some preliminary studies along these lines which will be published in a future paper.

The technique of "preparing" pigs as described by Thomson and Gledhill, and Doyle was not successful in our hands. However, the test was carried out in swine of Canadian origin which we have found to respond only poorly to the scarification test. We have tested many pigs from Canadian sources with uniformly mild lesions at the scarification sites and a mild hyperthermia. The elucidation of the mechanism of resistance in these animals is beyond the scope of this paper. The possibilities include individual resistance due to an existing or recent subclinical infection, or to a herd resistance due to hereditary factors. It does not seem likely that the former possibility is responsible. The possibility of herd resistance has been mentioned before. The point has been emphasized by Fortner [12] who apparently is attempting to select resistant families by breeding. On the other hand, the testing of the bacterins used was carried out in domestic swine from litters of proved susceptibility. If this precaution is observed, the test can be quite useful. It is hoped that, in the near future, a challenge procedure involving more severe reactions, including death, in an appreciable share of the control animals will be available.

Administration of cortisone in one study reported seems to increase, to some extent, the severity of response to scarification as demonstrated by more prolonged hyperthermia and death in 1 experimental animal. The erythema produced was not inhibited by the cortisone.

SUMMARY

1) In two studies, a total of 31 pigs were immunized with a single 5-cc. dose of an adsorbed *Erysipelothrix rhusiopathiae* bacterin, administered subcutaneously. These animals and 16 unvaccinated controls were challenged three weeks postvaccination by the scarification procedure of Fortner. The controls showed a febrile response together with severe swellings and erythema at the scarification sites. The vaccinated swine showed only a transient and minimal local response and no temperature rise.

2) Five pigs, "prepared" by previous vaccination with crystal violet vaccine followed by challenge with virulent hog cholera virus, showed only a febrile reaction to an intramuscular infection with *Ery. rhusiopathiae* culture. Five hog cholera-susceptible controls showed no significant difference from the "prepared" animals in their reaction to *Ery. rhusiopathiae* infection.

3) The administration of cortisone before and after experimental infection apparently caused the death of 1 of 4 pigs infected by the scarification procedure. Four pigs treated with cortisone, but infected by the intramuscular route, survived. Cortisone appeared to change the febrile response to infection with *Ery. rhusiopathiae*.

References

[1] Cooper, M. S., Personeus, G. R., and Choman, B. R.: Laboratory Studies on the Vaccination of Mice and Turkeys with an *Erysipelothrix Rhusiopathiae* Vaccine. Canad. J. Comp. Med., *18*, (1954): 83.

[2] Traub, E.: Immunisierung gegen Schweinerotlauf mit konzentrierten Adsorbatimpfstoffen. Monatsh. vet.-med., *10*, (1947): 165.

[3] Dinter, Z., Zur Schutzimpfung gegen Schweinerotlauf mit einer Adsorbatvakzine. Tierärztl. Umschau, *17-18*, (1948): 279.

[4] Fortner, J., and Dinter, Z.: Ist das Rotlaufbakterium der alleinige Erreger des Schweinerotlaufs? Z. Infekt.-Kr. Haustiere, *60*, (1944): 157.

[5] Shuman, R. D.: Swine Erysipelas Induced by Skin Scarification. Proc. Book AVMA, (1951): 153.

[6] Nardelli, L.: Sulla Preparazione di un Vaccino Formolato Adsorbito Concentrato contro il Malrosso dei Suini. Secondo Traub. Riv. di Med. Vet. e Zoo., *2*, (1950): 332.

[7] Bakos, K., and Lehnert, E.: A Study of the Immunizing Capacity of the Swedish Avirulent Swine Erysipelas Vaccine by Means of Percutaneous Infection. Nord. Vet.-med., *2*, (1950): 113.

[8] Basset, J.: Rouget du Porc; Quelques Précisions Concernant les "Vues Nouvelles" sur cette Maladie. Bull. Acad. vét. France, *23*, (1950): 103.

[9] Doyle, T. M.: Immunisation contre le Rouget du Porc. Off. Internat. des Épizoot., *38*, (1952): 140.

[10] Thomson, A., and Gledhill, A. W.: The Demonstration of the Protective Value of a Swine Erysipelas Vaccine in Pigs. Vet. Rec., *65*, (1953): 40.

[11] Doyle, L. P.: Rheumatoid Disease in Swine. Proc. U. S. Livestock San. A., (1949): 151.

[12] Fortner, J: Personal communication.

[13] Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F.: The Effect of a Hormone of the Adrenal Cortex (17 hydroxy-11-dehydrocorticosterone: Compound E) and of Pituitary Adrenocorticotropic Hormone on Rheumatoid Arthritis; Preliminary Report. Proc. Staff Meet. Mayo Clin., *24*, (1949): 181.

[14] Berlin, B. S., Johnson, C., Hawk, W. D., and Lawrence, A. G.: The Occurrence of Bacteremia and Death in Cortisone-Treated Mice. J. Lab. and Clin. Med., *40*, (1952): 82.

[15] Kligman, A. M., Baldridge, G. D., Rebell, G., and Pillsbury, D. M.: The Effect of Cortisone on the Pathologic Responses of Guinea Pigs Infected Cutaneously with Fungi, Viruses, and Bacteria. J. Lab. and Clin. Med., *37*, (1951): 615.

Experimental *Vibrio Fetus* Infection in Guinea Pigs
III. Pathogenicity Studies and the Effect of Guinea Pig Passage upon Agglutinogens

E. V. MORSE, D.V.M., Ph.D., and M. RISTIC, Dipl. Vet., D.V.M., M.S.

Madison, Wisconsin

CERTAIN bacteriological and pathological aspects of *Vibrio fetus* infection in guinea pigs were reported in previous publications.[4,5] This investigation deals with the pathogenicity of a number of strains of this organism and related vibrios isolated from domestic animals and man. The premise that residence in the laboratory animal might alter the antigenicity and suitability of the cells as agglutinogens is also considered.

MATERIALS AND METHODS

The vibrios which were isolated from the aborted fetuses of cattle and sheep were considered to be typical representatives of *V. fetus* on the basis of colonial and cellular morphology, while the two strains of human origin appeared to be this species. The swine dysentery organism was not classified; the vibrios which were isolated from the vagina or prepuce of cattle were probably *V. fetus* and were tentatively classified as such. The microorganisms were cultivated on solid agar and semisolid mediums under conditions which have been described previously.[4] The inoculum for the guinea pigs consisted of the growth from agar medium which was filtered, thrice washed with saline, and suspended in a sterile 0.85 per cent sodium chloride solution. The density of the material was standardized so that when 0.6 ml. was added to 10 ml. of saline a reading of 67 per cent light transmission in the Evelyn photoelectric colorimeter with the 660-μ filter was obtained. Four cultures of *V. fetus*, which were isolated from either bull semen or bovine vaginal mucus, produced such scant growth on the solid agar medium that the inoculums were prepared from the dense growth obtained on semisolid medium. The cells then were suspended in sterile saline, filtered through sterile glass wool, washed twice with sterile saline, and standardized in the same manner as employed for the cultures grown on solid medium.

The 5 guinea pigs employed in each trial were estimated to be in the fourth to eighth week of pregnancy. The inoculum consisted of 1 ml. of the saline Vibrio suspension and was administered by the intraperitoneal route.

Experimental Group 1.—Ten strains of *V. fetus*, all of which had been cultivated in the laboratory for three or more years, were used. Since these organisms might have proved to be nonpathogenic or of reduced pathogenicity, a total of two intraperitoneal inoculations were made on alternate days.

Experimental Group 2.—Twenty-one Vibrio strains were represented. Most of them had been isolated one to two years prior to this study. The pathogenicity of these organisms was ascertained by administering a single 1-ml. intraperitoneal inoculation of the standardized cell suspension.

The animals were examined several times daily after inoculation. As soon as practicable after the time of abortion or signs of impending death, the guinea pigs were killed, postmortem examinations were performed, cultures, and gram-stained smears

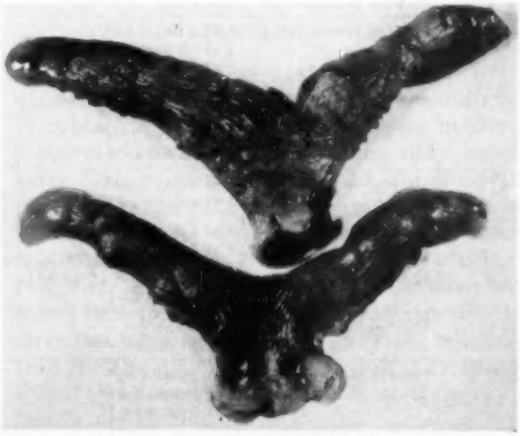


Fig. 1—Uteri of guinea pig showing extensive necrosis, hemorrhage, and edema. Top—abortion at three days after second intraperitoneal inoculation of Vibrio fetus, RL51. Bottom—abortion at one day after inoculation with same strain. Vibrios isolated from both organs.

From the Department of Veterinary Science, University of Wisconsin, Madison. Paper No. NS 150 published with the approval of the director of the Wisconsin Agricultural Experiment Station.

Dr. Ristic's present address: Department of Veterinary Science, University of Florida, Gainesville.

The authors thank Drs. A. H. Frank (Agricultural Research Service, U.S.D.A., Beltsville), W. R. Graham (Colorado A. & M. College), E. A. Tunnicliff (Montana State College, H. Stoenner (U.S.P.H. Laboratory, Hamilton, Mont.), W. N. Plastridge (University of Connecticut), E. O. King (Communicable Disease Center, Chamblee, Ga.), and Messrs. K. E. Price (Maryland Live Stock Sanitary Service, College Park) and H. E. Rhoades (University of Illinois) for sending Vibrio cultures for this investigation.

were made from the uterine contents. Several tubes of Albimi Brucella broth containing 0.1 per cent agar were inoculated with uterine material by means of a sterile capillary pipette. The cultures were incubated at 37 C. in an atmosphere of 10 per cent carbon dioxide and examined periodically for evidences of vibrios. The cultures which remained negative or were found to be contaminated were discarded after seven days. The animals which had not aborted or produced young were destroyed at sixteen days following inoculation. Autopsies were performed and uterine contents cultured for vibrios.

Antigens were prepared[2] for the serum agglutination test from the same material which was employed as inoculum for the guinea pigs. These antigens were designated as "original," as they had not been subjected to animal passage. As soon as a pure culture of the Vibrio strain in Albimi Brucella semisolid medium was obtained from the experimental animal, some of the growth was transferred to 16-ounce medicine bottles containing Albimi Brucella agar medium containing 2 per cent agar. The resultant growth was washed from the agar surface and antigen prepared.[2] This material was designated as "passed antigen." The original and passed antigens were compared as agglutinogens employing decimal dilutions of specific *V. fetus* antiserums of rabbit origin prepared against ten different strains.

RESULTS

All strains of *V. fetus* included in experimental group 1 produced abortion in 2 or more of the 5 inoculated animals. Pure cultures of the organism were isolated from 1 or more guinea pigs of each strain group following abortion. Abortions occurred as long as thirteen days and as early as the first day after inoculation. The vibrios were capable of residing in the pregnant uterus for periods up to fifteen days and could be demonstrated at the time of parturition or disposal by bacteriological culture. An abortion rate of 78 per cent was observed for the guinea pigs of this group. Severe illness and death were observed in 2 animals. The results are summarized in table 1.

Group 2 animals, which were given a single inoculation of known *V. fetus* strains, aborted at one to sixteen days. The abortion rate for the group was 62.5 per cent. *Vibrio fetus* was isolated in pure culture from 1 or more of the 5 animals which were inoculated with each strain. Parturitions occurred at one to sixteen days following inoculation and the organism was recovered

TABLE 1—Results Obtained Following Intraperitoneal Inoculation of Group 1 Guinea Pigs with Vibrio Fetus

Strain	Source	Termination and bacteriological findings			
		No. of abortions	No. of parturitions	No. destroyed	No. died
P 1	Aborted bovine fetus	3 at 1 day (+ + +)[1]		2 at 12 days (+ −*)[2]	
I 1	Aborted bovine fetus	1 at 1 day (+)[2] 1 at 10 days (+)[2] 2 at 13 days (+ −)[2]	1 at 8 days (−)[2]		
I 2	Aborted bovine fetus	2 at 1 day (+ +)[2] 1 at 2 days (+)[2] 1 at 3 days (+)[2] 1 at 10 days (−)[2]			
I 3	Aborted bovine fetus	1 at 1 day (+)[2] 1 at 4 days (+)[2]	1 at 15 days (−)[2]	1 at 3 days (+)[2]† 1 at 15 days (+)[2]	
RL 51	Aborted bovine fetus	4 at 1 day (+ + − C)[2] 1 at 3 days (+)[2]			
R 3	Aborted bovine fetus	4 at 1 day (−[1] +[2] +[2] +[2]) 1 at 7 days (+)[2]			
D 71	Aborted bovine fetus	3 at 1 day (− − −)[1] 1 at 10 days (+)[2]			1 at 2 days (C)[1]
M 2	Aborted bovine fetus	1 at 1 day (+)[1] 1 at 2 days (+)[1] 1 at 3 days (+)[2]	1 at 1 day (+)[2]	1 at 15 days (−)[2]	
M 3	Aborted bovine fetus	1 at 1 day (+)[2] 1 at 3 days (+)[2] 1 at 8 days (+)[2]	1 at 5 days (−)[2]	1 at 15 days (−)[2]	
543	Aborted ovine fetus	4 at 1 day (+ + + +)[2]. 1 at 9 days (+)[2]			

+ *V. fetus* isolated in pure culture from uterus. Each + indicates 1 animal; − *V. fetus* not isolated from uterus. C—Uterine culture contaminated; [1]—following first inoculation; [2]—following second inoculation; *—animal nongravid; †—animal destroyed when very ill.

from two or more uteri or fetuses. One of the strains, K, produced the death of 3 animals and the Vibrio organism was cultured from 1.

Most of the *V. fetus*-like strains which were originally cultured from bovine semen, bovine prepuce washings, or vaginal mucus gave results similar to those obtained with *V. fetus*, with the exception of three strains (9, 55, and 20). The inoculation of the later organisms resulted in one or no abortions and the vibrios were not reisolated from uterine material. The abortion rate for the group was 46 per cent. The swine dysentery Vibrio organism produced early abortion and was readily cultured from all animals. The two strains of human origin reacted similarly to *V. fetus* and were readily cultured from infected materials. The results of this experiment appear in table 2.

The gross pathological alterations observed in bacteriologically positive uteri were marked. The changes consisted of necrosis, extensive hemorrhage, and edema. The uteri usually contained a bloody, mucous exudate (fig. 1). *Vibrio fetus*, strain K, obtained from an aborted ovine fetus, produced generalized infections characterized by diffuse, fibrinous peritonitis, adhesions, hepatic necrosis, and acute cholecystitis.

Three strains of *V. fetus*, I3, M3, and 413, were significantly improved with respect to suitability as agglutinogens following residence in the guinea pig uterus for eight, fifteen, and sixteen days, respectively. This superiority was based upon the fact that the passed antigens were agglutinated at three or more tube dilutions higher than the original antigen when tested against ten specific *V. fetus* antiserums. The passed antigen of I3 proved better with six serums, that of M3 with five serums, and that of 413 with two serums. Agglutinogens prepared from four strains following a two- to twelve-day residence in the guinea pig proved to be slightly superior to original or nonpassed antigens. The passed antigens of these strains gave complete agglutination, while the original antigens were only partially clumped. Fifteen strains, which were reisolated from the host animal after one to fifteen days, showed no significant difference or improvement when employed as agglutinating antigens. Four strains, which prior to passage had appeared to be useless as antigens, were equally worthless following guinea pig passage.

Fig. 2—Liver of guinea pig given a single intraperitoneal inoculation of Vibrio fetus, K. Animal died at two days. Enlarged and inflamed gall bladder, areas of hepatic necrosis, and adhesions to the peritoneum are evident. Vibrio fetus isolated from the liver and uterus.

Discussion

A large number of viable cells, approximately 18×10^8, constituted the inoculum. It is possible that some of the severe inflammatory reactions and abortions may have been due, in part at least, to the foreign protein and not the pathogenic propensities of the vibrios. However, a considerable number of strains were capable of uterine residence and responsible for abortions at seven to sixteen days postinoculation. Such circumstances indicate a definite predilection for the gravid uterus and that in these cases the foreign protein reaction is certainly negligible.

Cultures which originated from porcine or human material readily produced early abortion following intraperitoneal inoculation. Similar results were obtained with many of the *V. fetus* strains originally isolated from the bovine vagina, prepuce, or semen. Three strains of this group were

TABLE 2—Results Obtained Following Intraperitoneal Inoculation of Group 2 Guinea Pigs with Vibrio Cultures

Strain	Source	Termination and bacteriological findings			
		No. of abortions	No. of parturitions	No. destroyed	No. died
2029	Aborted bovine fetus	1 at 1 day (+) 1 at 3 days (+)	1 at 1 day (+) 1 at 4 days (−) 1 at 6 days (−)		
981	Aborted bovine fetus	1 at 2 days (+) 1 at 3 days (+) 1 at 4 days (+) 1 at 12 days (+)	1 at 10 days (−)		
2221	Aborted ovine fetus	2 at 5 days (+ +) 1 at 7 days (+)	1 at 2 days (+) 1 at 6 days (−)		
53D134	Aborted ovine fetus	2 at 1 day (+ +) 2 at 2 days (+ +) 1 at 3 days (+)			
53D178	Aborted ovine fetus	1 at 2 days (+) 1 at 4 days (+) 1 at 5 days (+)	1 at 2 days (±)*	1 at 1 day (+)*	
412	Aborted ovine fetus	2 at 2 days (+ +) 1 at 5 days (−) 1 at 7 days (+)	1 at 1 day (±)*		
413	Aborted ovine fetus	1 at 2 days (+) 1 at 16 days (+)	1 at 16 days (−)	1 at 16 days (−)	1 at 4 days (C)
K	Aborted ovine fetus	2 at 1 day (+ +)**			3 at 2 days (+ C C)
D. L. F.	Bovine vaginal mucus	1 at 1 day (+) 1 at 9 days (+)		1 at 1 day (+)* 1 at 15 days (−)	1 at 1 day (C)
6	Bovine vaginal mucus	3 at 1 day (+ + +) 1 at 2 days (+)	1 at 5 days (−)		
8†	Bovine vaginal mucus	2 at 1 day (+ +) 1 at 3 days (+)	1 at 10 days (−) 1 at 12 days (−)		
9†	Bovine vaginal mucus	1 at 3 days (−)	1 at 1 day (−) 1 at 3 days (−) 1 at 5 days (−) 1 at 8 days (−)		
27	Bull semen	2 at 1 day (+ +) 1 at 5 days (+)	1 at 10 days (−) 1 at 15 days (−)		
54	Bull semen	1 at 1 day (+) 1 at 3 days (+) 1 at 5 days (+)	1 at 13 days (−) 1 at 14 days (−)		
56	Bull semen	1 at 2 days (+) 1 at 10 days (−) 1 at 14 days (+)	1 at 15 days (−) 1 at 15 days (−)		
55	Bull semen	1 at 3 days (−)	1 at 3 days (−) 1 at 5 days (−) 1 at 7 days (−) 1 at 8 days (−)		
20†	Bull semen		1 at 3 days (−) 1 at 4 days (−) 1 at 8 days (−) 1 at 10 days (−) 1 at 12 days (−)		
41	Bull preputial washing	2 at 2 days (+ +) 1 at 8 days (−)	1 at 11 days (−) 1 at 12 days (−)		
7298	Swine dysentery	4 at 1 day (+ + + +) 1 at 2 days (+)			
1134	Human blood	2 at 1 day (+ C) 2 at 2 days (+ +) 1 at 3 days (+)			
1154	Human blood	1 at 2 days (+) 1 at 3 days (+)	1 at 1 day (+) 1 at 6 days (C) 1 at 12 days (+)		

\+ *V. fetus* isolated in pure culture from uterus. Each + indicates 1 animal; ± *V. fetus* not isolated from uterus but observed in wetmount or stained preparation; − *V. fetus* not isolated.

C—Uterine culture contaminated; *—animal destroyed when ill; **—*V. fetus* isolated from liver of 1 animal; †—washed semisolid agar culture inoculated.

much less active. The abortions, which often occurred the day following inoculation, may have been secondary to the severe systemic inflammatory response induced by the agent or its protein. The fact that the organisms were isolated from the genital tract as early as the first postinoculation day does not indicate any selective affinity for the uterine or fetal tissues. However, the typical *V. fetus* strains which were isolated from cases of ovine and bovine abortion appeared to prefer the gravid uterus and often produced abortions at as long as seven to sixteen days following inoculation. A true predilection was apparent. Abortion rates for the *V. fetus* strains of 78 per cent (table 1) and 62.5 per cent (strains 2029-K, table 2) as contrasted to 46 per cent for the strains of mucous membrane or semen origin (strains DLF-41) indicate a significant difference in pathogenicity on the basis of origin. The latter two figures give a more equitable comparison since the animals of group 2 received a single inoculation, while those of group 1 received two inoculations. These findings may indicate that one is dealing with two or more species or that some pathogenic variant has been produced.

An antigenic relationship between the two strains of human origin and eight of 12 strains of *V. fetus* was demonstrable by the serum agglutination test. Since the *V. fetus* group exhibits serological heterogeneity,[1, 3] the evidence does not preclude that these organisms are separate species or at least antigenic variants of a common Vibrio type.

The passage of the vibrios in pregnant guinea pigs indicated that three of the cultures had been altered at least antigenically by such procedure. The possibilities are that either such residence altered the cells *per se* or that the less virulent members of the cell population were reduced or eliminated. Either of these two situations might well be responsible for the improvement noted in the antigen prepared from guinea pig passage Vibrio cells. Since the three strains which showed demonstrable agglutinogenic superiority had resided eight, fifteen, and sixteen days in the host, it follows that such a sojourn might well favor this change. The premise that such procedures would alter antigens by either producing or eliminating variant types of *V. fetus* warrants further attention.

SUMMARY

The pathogenicity of 31 strains of *Vibrio fetus* or *V. fetus*-like organisms for pregnant guinea pigs was investigated. The majority produced abortion, and isolation of the agent from genital tract or fetal materials at the time of autopsy was accomplished. Residence in the host for periods of sixteen days was observed. The gross pathological changes seen in the infected animals are discussed.

Three of the strains, following guinea pig passage, proved to be more suitable as antigens for the serum-agglutination test than were the cells which were not subjected to the passage procedure. The evidence may indicate that either variant types are being eliminated or that antigenic variants are produced or allowed to multiply during parenteral residence in the guinea pig.

References

[1] Marsh, H., and Firehammer, B. D.: Serological Relationships of Twenty-Three Ovine and Three Bovine Strains of *Vibrio Fetus*. *Am. J. Vet. Res.*, *14*, (1953): 396-398.

[2] Morse, E. V., Ristic, M., Robertstad, G. W., and Schneider, D. W.: Cross-Agglutination Reactions Among Brucella, Vibrio, and Other Microorganisms. Am. J. Vet. Res., *14*, (1953): 324-327.

[3] Price, K. E., Poelma, L. J., and Faber, J. E.: Preliminary Report on Serological Relationships Between Strains of *Vibrio Fetus*. Vet Med., *48*, (1953): 355-358.

[4] Ristic, M., and Morse, E. V.: Experimental *Vibrio Fetus* Infection in Guinea Pigs. I. Bacteriological Aspects. Am. J. Vet. Res., *14*, (1953): 399-404.

[5] Ristic, M., Wipf, L., Morse, E. V., and McNutt, S. H.: Experimental *Vibrio Fetus* Infection in Guinea Pigs. II. Pathological Aspects. Am. J. Vet. Res., *15*, (1954): 137-139.

The Secondary Immune Response in Chickens Revaccinated with Inactivated Newcastle Disease Virus Vaccine

M. S. HOFSTAD, D.V.M., Ph.D.

Ames, Iowa

The purpose of this investigation was to extend the previous observations regarding the secondary immune response to inactivated Newcastle disease virus vaccine, and to determine whether the initial dose could be given at 2 days of age rather than at 3 weeks or later as had been done in previous experiments.

Literature Cited

In previous reports[1,2] data were presented to indicate an increased resistance in chickens to Newcastle disease virus (NDV) when two doses of inactivated vaccine were given at least nine weeks apart and preferably longer.

Brandly *et al.*[3] observed that a group of chickens revaccinated after an eight-week interval had a considerably lower incidence of challenge infection than did lots vaccinated once or lots revaccinated at shorter intervals.

Waller and Gardiner[4] have shown that chickens have greater resistance to challenge when a second dose of inactivated Newcastle disease vaccine is given thirteen weeks following the initial dose.

Procedure

Six experimental groups were made from 357 White Leghorn chickens of both sexes (table 1). The chicks were hatched from eggs laid by hens which were susceptible to Newcastle disease. They were housed in the same house for the first four months, and at the end of that time group 6 was moved to another house until the completion of the experiment.

An alumina-gel, formalin-inactivated, commercial vaccine was used. Distributed through the six groups were 187 chicks which were given the first dose of 0.5 ml. of vaccine at 2 days of age (table 1). At 4 weeks of age, 25 chickens from group 2 were given a 1-ml. dose of vaccine for the first time. These were to serve as once-vaccinated controls for the chickens which were given a second dose at the same age. At the same time, the 25 chickens which had been vaccinated at 2 days of age in group 2 were given a second 1-ml. dose of vaccine. Eleven chickens were left as unvaccinated controls in group 2. At 8, 12, 16, and 20 weeks of age, groups 3, 4, 5, and 6, respectively, were handled similarly to groups 2 (table 1). After the chickens in each group were vaccinated they were kept in isolated pens the following four months until challenge.

Each group was challenged with the GB-Texas-1948 strain of Newcastle disease virus four months after the second or final dose of vaccine had been given. The challenge dose, dropped into both nostrils, was 0.1 ml. of a 1:100 dilution of pooled infected allantoic fluid. The interpretation of the challenge was based on results of attempts to isolate the virus from the blood, spleen, or lung ninety-six hours following challenge as described previously.[1,2] In one half of the chickens in groups 5 and 6, the challenge dose was increased to 0.1 ml. of a 1:10 dilution. On the day of each challenge the inoculum was titrated in embryonating eggs and also in chickens 6 to 9 weeks old by the method previously described.[1] The results of these titrations are given in table 2.

Results

The results of challenge of each of the six groups are presented in table 2, and in figure 1. Four months after a single 0.5-ml. dose of vaccine, challenge results indicated 4 of 26 chickens were immune in group 1. A second dose of 1 ml. given to 23 chickens in group 2 at 4 weeks of age did not result in a greater number of resistant individuals since 47.8 per cent of these were immune as compared to 52 per cent protection for the once-vaccinated chickens. In group 3, the second 1-ml. dose

TABLE 1—Plan of Experiment

Group	No. Chickens* unvac.	No. Chickens* vac.	Age at time of first vac.	Age at time of second vac.	Age in weeks at challenge
1	11	26	2 days		16
2	11	25	2 days	4 weeks	20
		25	4 weeks		20
3	11	27	2 days	8 weeks	24
		20	8 weeks		24
4	10	36	2 days	12 weeks	28
		21	12 weeks		28
5	10	38	2 days	16 weeks	32
		21	16 weeks		32
6	10	35	2 days	20 weeks	36
		20	20 weeks		36

* Numbers given include 15 chickens which died from miscellaneous causes during the course of the experiment.

From the Veterinary Research Institute and the Animal Pathology Section of the Agricultural Experiment Station, Iowa State College, Ames. The author expresses appreciation for the cooperation given by the staff of the Veterinary Research Institute, to Dr. Arne W. Nordskog of the Poultry Husbandry staff and to Miss Francais Weddle for technical assistance.

of vaccine, given at 8 weeks, likewise failed to increase significantly the resistance to challenge (66.6 %) when compared with the once-vaccinated control birds (63.2 %).

In group 4, a second dose given at 12 weeks of age resulted in a greater immunity than that which developed among the birds vaccinated once at 12 weeks of age. Of the twice-vaccinated chickens 87.9 per cent resisted challenge as compared to 28.6 per cent for the once-vaccinated individuals. Part of this difference probably was due to a lesser immunity from one dose of vaccine administered to chickens older than 8 weeks (groups 4, 5, and 6).

The chickens in groups 5 and 6 responded to challenge with almost identical results indicating that a second dose given to chickens sixteen or twenty weeks after the initial dose greatly enhanced the birds' resistance to NDV. At the end of four months, 94.7 per cent and 94.4 per cent, respectively, of the twice-vaccinated chickens in groups 5 and 6 resisted challenge infection as compared to 20 per cent among the once-vaccinated control chickens.

When a tenfold increase in challenge virus was given to half of the birds in groups 5 and 6, the percentage of birds with immunity was decreased by approximately 40 per cent in each group of twice-vaccinated chickens (table 2). However, the increased challenge dose did not lower the percentage of immune individuals among the once-vaccinated chickens. While not anticipated in the original plan of the experiment, twenty-five blood samples were collected at the time of challenge from the twice-vaccinated chickens in group 6. The serum samples were tested for virus infectivity neutralizing antibody levels using the GB challenge virus. Three serums had 1,000 or less neutralizing doses. Two of these birds failed to resist the increased challenge dose. Two serums had between 10^3 and 10^4 neutralizing doses and both of these chickens failed to resist the increased challenge dose. Three serums had between 10^4 and 10^5 neutralizing doses and 1 of these failed to resist the increased challenge dose. Eleven serums had between 10^5 and 10^6 neutralizing doses. Four of these chickens were challenged with the 10^{-2} dilution of virus and 1 failed to resist. Seven of the chickens were challenged with the tenfold increase in dose and 2 failed to resist the challenge. Six serums had 10^6 or more neu-

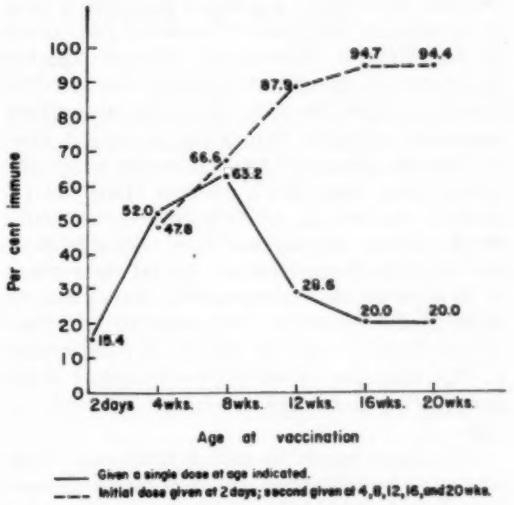


Fig. 1—Percentage of vaccinated chickens immune to intranasal challenge with 0.1 ml. of 10^{-2} dilution of Newcastle disease virus infected allantoic fluid.

TABLE 2—Results of Challenging Vaccinated and Unvaccinated Control Chickens with Newcastle Disease Virus

Group	Chickens infected by challenge/number challenged			Per cent immune		Titer of challenge virus in	
		vaccinated		vaccinated			
	unvaccinated	once	twice	once	twice	embryos	chickens
1	11/11	22/26		15.4		$10^{-6.5}$**	$10^{-6.33}$†
2	10/10	12/25	12/23	52.0	47.8	$10^{-9.0}$	$10^{-5.75}$
3	11/11	7/19	8/24	63.2	66.6	$10^{-9.3}$	$10^{-4.86}$
4	9/9	15/21	4/33	28.6	87.9	$10^{-9.0}$	$10^{-5.0}$
5	5/5	8/10	1/19	20.0	94.7	$10^{-9.5}$	$10^{-5.67}$
5*	4/4	8/10	8/18	20.0	55.6		
6	5/5	8/10	1/18	20.0	94.4	$10^{-9.0}$	$10^{-5.86}$
6*	4/4	8/10	8/17	20.0	52.9		

* This half of groups 5 and 6 birds received a tenfold greater challenge dose.
** $10^{6.5}$ embryo lethal doses per 0.05 ml. dose.
† $10^{6.33}$ chicken infective doses per 0.1 ml. dose.

tralizing doses and all these birds resisted the challenge.

DISCUSSION

The results presented confirm the observations previously reported that the degree of immunity obtained following two doses of inactivated Newcastle disease vaccine depends to a great extent upon the interval between doses. In this study, the secondary immune response following a second dose of vaccine given at twelve weeks after the initial dose was much greater than the response following a dose given at eight weeks after the initial dose. In addition, the data indicate that an initial dose given at 2 days of age prepares the birds for an anamnestic immune response to a subsequent dose of vaccine which is comparable to the response observed previously [1] when the initial dose was given at 3 weeks of age.

The response of 4- and 8-week-old chickens to a single dose of Newcastle disease vaccine is interesting. The poor response of 2-day-old chicks was expected, but the resistance to infection of 52.0 and 63.2 per cent of the birds given a single dose of vaccine at 4 and 8 weeks of age was not anticipated in view of the relatively poor immunity in the chickens vaccinated once at 12, 16, and 20 weeks of age. The differential response of chicks vaccinated once at 4 weeks or at 8 weeks of age compared with that among birds younger or older at first vaccination is statistically highly significant ($P < .01$) based on a *chi*-square test.[5] Additional information is needed on this phase of Newcastle disease immunization of individual groups of birds with inactivated vaccines. The difference in immunity between the twice-vaccinated chickens in groups 4, 5, and 6 is not significant.

The response of birds in groups 5 and 6 to a tenfold increase in challenge virus indicates that the challenge dose can change the apparent results considerably. It emphasizes the importance of defining an adequate challenge dose. Had a dosage of 0.1 ml. of a 10^{-1} dilution of challenge virus been used throughout the experiment, the results might have been different. However, since all controls became infected, and in groups 5 and 6, 80 per cent of the once-vaccinated became infected, it is believed that the smaller challenge dose was adequate in this experiment. While the results of testing the serums from group 6 birds were variable, only two serums had less than 1,000 neutralizing doses. Following challenge the virus was recovered from 1 of these birds but not from the other. The high titer of neutralizing antibody in the other serums is in agreement with the finding that 94.4 per cent of the birds in group 6 resisted challenge to the 10^{-2} dilution of virus. However, when the tenfold increase in challenge dose was given to 14 of the chickens whose serums were tested, 7 of the 14 failed to resist the challenge indicating that the challenge was either overwhelming or that the virus is permitted to multiply in the respiratory system even though the serum possesses high antibody levels.

SUMMARY

Six groups of chickens were used to determine the secondary immune response to a "booster" dose of inactivated Newcastle disease vaccine. Controls consisted of unvaccinated chickens and chickens vaccinated once at the time the second dose was given. The initial dose was given at 2 days of age to the twice-vaccinated group and the second dose was given at intervals of 4, 8, 12, 16, and 20 weeks, respectively. Four months after the last dose of vaccine each group was challenged by intranasal inoculation of the GB-Texas-1948 strain of Newcastle disease virus. Of the chickens revaccinated at 12, 16, or 20 weeks of age, 87.9, 94.7, and 94.4 per cent, respectively, were resistant to the challenge as compared to 28.6, 20.0, and 20.0 per cent of the once-vaccinated control chickens. Chickens vaccinated once at 4 or 8 weeks of age possessed a significantly greater immunity than groups of birds vaccinated once at 2 days, 12 weeks, 16 weeks, or 20 weeks of age.

References

[1] Hofstad, M. S.: Immunization of Chickens Against Newcastle Disease by Formalin-Inactivated Vaccine. Am. J. Vet. Res., *14*, (1953): 586-589.

[2] Hofstad, M. S.: A Method of Evaluating Immunity Following Vaccination of Chickens with Inactivated Newcastle Disease Vaccine. Am. J. Vet. Res., *14*, (1953): 590-593.

[3] Brandly, C. A., Moses, H. E., Jones, E. E., and Jungherr, E. L.: Immunization of Chickens Against Newcastle Disease. Am. J. Vet. Res., *7*, (1946): 307-332.

[4] Waller, E. F., and Gardiner, M. R.: Newcastle Disease: Response to Formalized Vaccine. Poult. Sci., *32*, (1953): 405-411.

[5] Snedecor, G. W.: Statistical Methods. Iowa State College Press, Ames, Iowa, (1946): 206.

The Therapeutic Effect of Streptomycin on Infectious Coryza of Chickens Caused by *Hemophilus Gallinarum*
I. Blood Serum Levels of Streptomycin in Chickens

S. BORNSTEIN, B.S., D.V.M., and Y. SAMBERG, Dr. Vet. Med.

Tel Aviv, Israel

DEBLIECK [4,5] was apparently the first investigator to report good results following intramuscular injections of streptomycin and dihydrostreptomycin (sulfate) in the treatment of chickens suffering from infectious coryza type II, that is, a coryza caused by coccobacilliform bodies.[3] This limited study has not been followed with further publications in the veterinary literature and, to the best of our knowledge, the only other report on this subject comes from Italy.[9] However, according to local poultrymen returning from visits to Holland, the treatment of coryza with streptomycin has become a routine procedure in that country. From there, this method of therapy has spread to Israel with surprising success.

The purpose of this study was to ascertain whether the accepted use of a very high (and almost uneconomical) dosage of streptomycin was justified, and to discover a few characteristics about the type [3] of coryza, and its pathogen, prevalent in Israel. In order to establish the correct dosage for an efficient treatment of infectious diseases with antibiotics, it is necessary to determine the sensitivity of the pathogen to this antibiotic and the blood levels obtainable with the latter. Consequently, the first report of this series will deal with the blood serum levels of streptomycin in chickens.

LITERATURE CITED

In veterinary medicine, blood serum levels of streptomycin have been determined for horses,[7,11] cattle,[6,11,13] sheep,[6] goats,[8] and dogs;[10,11,18,22] and in laboratory animals for monkeys,[15,22] rabbits,[15] guinea pigs,[15] and mice.[15,20,23] However, no systematic determinations of such blood levels have been made for poultry. According to the literature at our disposal, the only approach to such a study was made by Huebner *et al.*[12] in turkeys, who determined their blood levels after implantation of penicillin-streptomycin pellets and, for comparison, after injecting 25 mg., subcutaneously.

From the Government Poultry Field Laboratory, Livestock Department, Ministry of Agriculture, Tel Aviv, Israel.

Published with the approval of the director of the Livestock Department, Israel Ministry of Agriculture.

MATERIALS AND METHODS

Antibiotic.—All injections were made with dihydrostreptomycin (sulfate) of various brands. No significant differences in the serum level response of chickens could be correlated with the use of products of various foreign origins or of local packaging. The various doses of streptomycin were injected as a 1-cc. solution diluted with sterile distilled water. According to the recent report of Gratzl *et al.*,[11] this might have been a mistake, because these workers found that the duration of blood concentrations is not only dependent on the dosage of streptomycin but also on the concentration of the injected solution. Thus, in horses receiving a dose of 5,000 units per kilogram of body weight, a solution containing 50 per cent streptomycin gave detectable blood concentrations for twenty-eight hours, while one of 9 per cent, for only ten hours. A similar situation was described for cattle and for dogs, in which the same dosage gave increasingly longer blood concentrations when the concentration of the injected solution rose from 9 per cent to 16 per cent, to 33 per cent and to 50 per cent. On this basis, our injected streptomycin solutions varied from 2.5 per cent for the dose of 25,000 units (μg.) to 20 per cent for the 200,000-unit dose.

Chickens.—The chickens used (except for the young ones of table 2) were 12 adult Leghorn hens kept in cages in the laboratory. Their weight varied from 1.25 kg. to 2.25 kg., half of them fell within the range of 1.50 to 1.80 kg., and the mean weight was 1.70 kg.

Collection of Serum.—Blood samples were collected into sterile test tubes from the wing vein by means of a slight incision with a sterile razor blade after suitable preparation of the site (plucking of feathers and rubbing with alcohol). Several minutes after the slanted clot had formed, it was freed from the tube walls by energetic tapping of the test tube against the palm of the hand. The free clot was suspended by squeezing its thin end between the cotton stopper and the glass wall of the tube. The latter was placed into a bacteriological incubator for one hour and left over night in a household-type refrigerator. In this manner, a maximum of nonhemolyzed serum was obtained from relatively small blood samples

without resorting to centrifugation. Many sterility tests performed on such serum samples, which were collected in this manner for the first time in connection with the determination of penicillin-blood concentrations,[21] proved that this method was very satisfactory in spite of its crudeness. The same bird was never tested more than once a week, and usually less frequently.

Assay Procedure.—Except for two slight technical modifications, exactly the same assay method was used as has been described in a previous paper for the determination of penicillin-serum levels.[21] This simple method proved itself suited for the determination of streptomycin concentrations in blood serum, yielding end points which were more clear-cut than those obtained with penicillin. A later survey of the literature showed that this method is similar to the one described by Price and co-workers,[19] except for the test organism. Our procedure was as follows. Each test consisted of a series of tubes sufficient to insure an end point, the first tube of each series being empty and the others containing 0.5 cc. of sterile, plain, nutrient broth. Into each of the first and second tubes was placed 0.5 cc. of the serum sample; serial twofold dilutions were made through the tubes in the series starting from the second tube. For the control series, which was included in each batch of tests, commercial streptomycin was diluted in sterile, distilled water to a concentration of 4 units per cubic centimeter. As with the serum samples, doubling dilutions were made with this standard streptomycin solution. To each tube of the standard and of the serum series, 0.5 cc. of inoculum was added.

Test Organism.—Since this is a comparative test, any organism sensitive to the bacteriostatic action of streptomycin is suitable. Thus, for instance, Price and co-workers[19] used *Bacillus circulans*, Hughes and Farmer[18] used Friedlander's Bacillus, and we continued with our strain of *Staphylococcus aureus* (of mastitis origin). Since the action of streptomycin is influenced by such factors as size of inoculum, physical state of medium, duration of incubation, et cetera,[1, 2, 15, 16, 17] some preliminary tests were performed in order to determine the optimum dilution of the organism in the inoculum. As a matter of fact, inconsistent differences of one tube each were found between dilutions of 1:100, 1:10,000, and 1:1,000,000. For technical reasons, it was decided to use an inoculum consisting of a 10^{-4} dilution in plain broth of an eighteen-hour broth culture.

Interpretation of Tests.—Readings were made after twenty-one hours of incubation at 36 to 37 C., and the last tube in which no bacterial growth was perceptible was considered as end point. After direct comparison with the standard series, this titer was then expressed as units (μg.) of streptomycin per cubic centimeter of serum. The above test detected potencies as low as 0.12 units of streptomycin per cubic centimeter of serum (and sometimes even 0.06 u./cc.); therefore, values of 0.0 in the tables mean more accurately, < 0.12. The sensitivity of this test is completely adequate, because a minimum of 3 to 6 units per cubic centimeter of serum are considered to be necessary to overcome most of the susceptible organisms.[22]

Serological titrations may at times show unusual and extreme values which easily distort the arithmetic mean. Consequently, all averages are computed and expressed as "median," an average which is not influenced by single or exceptional values.

RESULTS AND DISCUSSION

Table 1 summarizes the serum level response of adult chickens to various doses of aqueous streptomycin injected intramuscularly. Obviously, the serum level responses of different chickens receiving the same dosage of streptomycin were quite uniform for corresponding assay periods, and the ranges in blood concentrations did not exceed fourfold limits. Similar uniform results were reported by Doll and co-workers.[7] Twofold increases in dosage did not consistently double the serum concentration.

When table 1 is compared with the corresponding table of a previous paper of this laboratory on penicillin blood levels

TABLE 1—Serum Levels Following Intramuscular Injection of Streptomycin (Six Trials per Dosage)

Dosage		Results expressed as	Units of streptomycin per cc. of serum at specified hours after injection (hours)						
Units per chicken	Units per kg.*		½	1	3	6	8	12	24
25,000	15,625	range	2-4	2-4	1-2	0.25-1.0	0.25-0.5	0.0†-0.12	
		median**	2	4	1.5	0.5	0.25	0.12	
50,000	31,250	range	4-4	4-16	1-2	0.5-1.0	0.25-0.5	0.12-0.25	0.0-0.0
		median	4	4	2	0.75	0.37	0.18	0.0
100,000	62,500	range		16-64	4-16	1.0-2.0	0.5-1.0	0.25-0.5	0.0-0.12
		median		16	8	2.0	1.0	0.5	0.0
200,000	125,000	range		16-64	4-16	1-4	0.5-2.0	0.25-1.0	0.0-0.25
		median		32	12	4	2.0	0.75	0.18

* Based on median weight of 1.6 kg. per chicken;
** Based on six trials; † 0.0 = < 0.12.

in chickens,[21] one major difference becomes immediately apparent between the serum level responses of chickens to intramuscular streptomycin and penicillin. The latter is absorbed very fast into the blood stream, yields maximal blood concentrations ten to twenty minutes after the injection and, after one hour, these concentrations are only one quarter of the maximal ones. Streptomycin, on the other hand, is absorbed slowly and reaches maximal levels in the chicken blood approximately one hour after administration (not enough determinations were made half an hour after injection, and none two hours later, to fix more definitely the time of maximal blood concentrations). On a whole, the serum levels three hours after the injection are approximately one half, and those after six hours about one eighth, of the one-hour level.

TABLE 2—Blood Levels in Chickens of Different Weights Following Intramuscular Injections of 100,000 Units of Streptomycin per Bird (Units per Cubic Centimeter of Serum)

Weight	Dosage	Blood levels after hours		
(kg.)	(u./kg.)	1	3	6
0.300	333,333	16	16	2
0.350	285,714	32	16	2
0.450	222,222	64	8	1
0.550	181,818	32	16	4
0.750	133,333	64	16	8
0.850	117,647	32	32	2
1.000	100,000	32	32	....
1.350	74,074	16	4	....
1.500	66,666	16	8	2
1.500	66,666	16	8	1
1.600	62,500	16	8	2
1.600	62,500	32	4	2
1.800	55,000	64	8	1
1.900	52,631	32	16	2

Not only is streptomycin absorbed slowly into the blood stream, as compared with penicillin, but its relatively slow elimination from the body makes for a longer maintenance of blood concentrations, even without the aid of special agents for the retardation of its absorption into, and its excretion from, the blood. In comparing these two antibiotics in dogs, Manuila [16] shows that, under certain conditions, 60 per cent of penicillin is found in the urine within one hour after its injection and 100 per cent within four hours, while with streptomycin, 50 per cent of the injected dosage is found in the urine within two hours; at the end of twenty-four hours 80 per cent was thus eliminated.

In man, there is a tendency to slightly higher values two hours after the injection of streptomycin than after one hour,[14] but in horses [7] there is a reverse tendency. In cattle,[13] maximal blood concentrations are obtained after two hours, while in sheep,[6] after two hours they are the same or less than after one hour. The dog seems to behave exceptionally in this respect by showing its highest blood concentrations one fourth to one half hour after the injection.[18,23] All the above species, as is the case with chickens, maintain approximately half of their maximal serum level to about three hours after the injection.

It is customary to express the dosage of antibiotics as units of the material per unit of body weight, that is in the present case, units of streptomycin per kilograms of body weight. However, in arranging the results for table 1, no consistent differences were noted between chickens of different weights. These observations were enlarged

TABLE 3—Comparison of Serum Level Responses of Different Species Following Intramuscular Injections of Streptomycin

Species	Dosage (u./kg.)	Author	Units streptomycin per cc. serum, after injection (hours) (hr.) 1	2	3	4	6
Man	3,000*	Kolmer [14]	5-10	5-8	4-6	2-3	1-2
Horse	4,000	Doll [7]	8	6	4	....	....
Sheep	4,000	Doll [7]	8	6	3.8	2.6	1.3
Dog	4,000	Stebbins [23]	5	4	2	....	....
Chicken	31,000	Table 1	4	....	2	....	0.75
Man	7,000*	Kolmer [14]	12-15	15-20	10-15	10-12	6-8
Man	7,000*	Rake [20]	10	20	20	20	....
Man	8,500*	Zintel [24]	17	18	18	16	9
Horse	8,000	Doll [7]	16	12	8	....	....
Cow	11,000	Edwards [8]	....	20.5	....	20.5	10.2
Sheep	8,000	Doll [7]	16	13	12	6.5	3.7
Dog	8,000	Molitor [18]	16	10	8	4	....
Chicken	62,000	Table 1	16	....	8	....	2

* Assuming 70 kg. of body weight per patient.

by administering 100,000 units per bird to chickens weighing less than 1.250 kg. (the lowest weight of our test birds). The results obtained are summarized in table 2.

According to this table, birds weighing less than 1 kg. have a tendency to have slightly higher blood levels than those weighing more than 1 kg. For instance, the median blood level one hour after injection is 32 units per cubic centimeter for the lower weight group and 16 units per cubic centimeter for the higher one (16 and 8 u./cc., and 2 and 2 u./cc. after 3 and 6 hours, respectively). However, in individual birds this difference does not necessarily hold true. Among the chickens of table 2, those weighing 0.35 kg. and 1.90 kg. gave identical serum level responses, as did those weighing 0.45 kg. and 1.80 kg. Consequently, within the approximate weight range of ⅓ to 2 kg., it is impossible to express the dosage of streptomycin as units per body weight, and the preferable way must be as units per bird.

These results did not come as a surprise to us, because an identical situation was met and reported with intramuscular injections of penicillin in chickens.[21] In the latter study, 75 birds ranging in weight from 45 to 2,000 Gm. were divided into five weight groups and part of every group was injected with one of three different doses (units per bird); that is, each dose was tested on 5 birds per weight group. Within the limits of 200 Gm. to 2 kg. "chickens receiving the same dose of penicillin, expressed as units per bird, gave the same serum level response, although this dosage, if expressed as units per kilogram of body weight, might vary up to tenfold."

The determinations which went into making up table 2 were made rather incidentally and over an extended period; therefore, the close range of corresponding values obtained tends to emphasize the reliability of these results.

Table 3 attempts to compare the serum level response of various species to the intramuscular injection of streptomycin. Such a comparison can not be accurate, because there is no common basis for the different studies here included in the single framework of one table, which is intended merely as a rough expression of tendencies. Nevertheless, it seems rather obvious that for equal doses (expressed as u./kg. of body weight) of intramuscular streptomycin, the serum level responses of men, horses, cows, sheep, and dogs are relatively similar. On the other hand, chickens show themselves as very inefficient converters of intramuscular streptomycin into serum streptomycin, and they require approximately an eightfold dosage (in u./kg.) in order to obtain serum concentrations of the same order as the above species.

SUMMARY

A simple dilution method for the determination of streptomycin in the blood serum of chickens has been described.

The serum level responses of adult Leghorn hens to intramuscular injections of 25,000 to 200,000 units of streptomycin per bird are presented. Serum concentrations in different chickens, which received the same dosage and for corresponding assay periods, were rather uniform.

In contrast to penicillin, streptomycin is absorbed into the blood stream rather slowly, and its equally slow elimination enables a longer duration of the serum level period.

Within the weight range of ⅓ kg. to 2 kg., approximately the same serum level response is obtained from chickens receiving the same intramuscular dosage of streptomycin, expressed as units per bird, although these doses might differ sixfold if expressed as units per kilogram of body weight. The latter (traditional) manner of expressing the dosage of streptomycin is, therefore, unsuitable for chickens.

In comparison with other species, chickens require about an eightfold dosage (expressed as u./kg. of body weight) in order to reach equivalent serum concentrations of streptomycin.

References

[1] Alexander, H. E., and Leidy, G.: Influence of Streptomycin on Type B *Haemophilus Influenzae*. Science, *104*, (1946): 101-102.

[2] Chang, K. H., and Stafseth, H. J.: Influence of Various Factors on the Bacteriostatic and Bactericidal Action of Streptomycin on *Salmonella Pullorum*. Poult. Sci., *29*, (1950): 130-138.

[3] DeBlieck, L.: Het Tegenwoordig Standpunt Inzake Coryza-Infectiosa-Gallinarum. Tijdschr. Diergeneesk., *73*, (1948): 955-962.

[4] DeBlieck, L.: De Therapie van Coryza Infectiosa Gallinarum Type II (Nelson) met Streptomycine. Tijdschr. Diergeneesk., *75*, (1950): 538-541.

[5] DeBlieck, L.: The Treatment of Coryza Infectiosa Gallinarum Type II (Nelson) with Streptomycin. Vet. Rec., *62*, (1950): 787.

[6] Doll, E. R., and Wallace, M. E.: Dosage and Blood Level of Streptomycin in Sheep. J.A.V.M.A., *113*, (1948): 55-58.

[7] Doll, E. R., Wallace, M. E., and Hull, F. E.: Serum Levels of Streptomycin in Horses and Sheep. Kentucky Agric. Exptl. Sta. Bull. 548, 1950.

[8] Edwards, S. J., and Haskins, M. D.: The Determination of Antibiotic Levels in Blood and in Milk Following Parenteral and Intramammary Injection. J. Comp. Path. Therap., *63*, (1953): 53-67.

[9] Galli, G.: Effect of Streptomycin on Infectious Coryza of Poultry (In Italian). Clin. Vet., *76*, (1953): 33-35.

[10] Graham, B. E., VanderBrook, M. J., and Kuizenga, M. H.: Preliminary Studies on the Absorption and Excretion of Streptomycin in Dogs. Science, *103*, (1946): 164-165.

[11] Gratzl, E., Jaksch, W., and Szumlakowsky, R.: Der Streptomycin-, Terramycin-, und Aureomycin-Blutserumspiegel bei Pferd, Rind und Hund. Proc. Fifteenth Internat. Vet. Cong., *2*, (1953): 1108-1113.

[12] Huebner, R. A., Elias, Wm. F., and Merrion, H. J.: Antibiotic Blood Levels in Turkeys Following Penicillin-Streptomycin Implantation. J.A.V.M.A., *121*, (1952): 187-189.

[13] Hughes, D. L., and Farmer, R. K.: Studies on Streptomycin in Relation to its Possible Uses in Veterinary Practice. Vet. Rec., *62*, (1950): 265-270.

[14] Kolmer, J. A.: Penicillin Therapy Including Streptomycin, Tyrothricin, and Other Antibiotic Therapy. 2nd ed. D. Appleton-Century Co., Inc., New York, 1947.

[15] Kornegay, G. B., Forgacs, J., and Henley, T. F.: Studies on Streptomycin. J. Lab. Clin. Med., *31*, (1946): 523-534.

[16] Manuila, L.: La Streptomycine. F. Rouge, Lausanne, 1950.

[17] McNeil, E., and Hinshaw, W. R.: The Effect of Streptomycin on *Pasteurella Multocida in Vitro*, and on Fowl Cholera in Turkeys. Cornell Vet., *38*, (1948): 239-246.

[18] Molitor, H.: Cited by Manuila.[16]

[19] Price, C. W., Nielsen, J. K., and Welch, H.: The Estimation of Streptomycin in Body Fluids. Science, *103*, (1946): 56.

[20] Rake, G., and Donovick, R.: The Use of the Mouse in Studies on Streptomycin. Proc. Soc. Exptl. Biol. Med., *64*, (1947): 22-25.

[21] Samberg, Y., Bornstein, S., and Rautenstein-Arazi, A.: Blood Serum Levels of Penicillin in Chickens. Refuah Vet., *10*, (1953): 37-50

[22] Scheidy, S. F.: Some Sulfonamides and Antibiotics in Veterinary Practice. North Am. Vet., *32*, (1951): 245-250.

[23] Stebbins, R. B., Graessle, O. E., and Robinson, H. I.: Studies on the Absorption and Excretion of Streptomycin in Animals. Proc. Soc. Exptl. Biol. Med., *60*, (1945): 68-72.

[24] Zintel, H. A., Flippin, H. F., Nichols, A. C., Wiley, M. M., and Rhoads, J. E.: Studies on Streptomycin in Man. Am. J. M. Sci., *210*, (1945): 421-430.

The Therapeutic Effect of Streptomycin on Infectious Coryza of Chickens Caused by *Hemophilus Gallinarum* II. Isolation and Culture of *Hemophilus Gallinarum*, and Some of Its Biochemical Reactions

S. BORNSTEIN, B.S., D.V.M., and Y. SAMBERG, Dr. Vet. Med.

Tel Aviv, Israel

IN THE previous paper,[2] it was stated that one of the purposes of this study was to investigate the necessity for the very high doses of streptomycin which are in use at present in this country. This required (*a*) a determination of blood levels obtainable with streptomycin in chickens, and (*b*) a study of the sensitivity of the pathogen to this antibiotic. The blood levels of streptomycin have been presented;[2] however, the second requirement is complicated by the fact that *Hemophilus gallinarum* is a bacterium which is difficult to isolate in pure culture and not less difficult to grow and maintain with regular laboratory methods, two prerequisites which must precede any attempt to study the sensitivity of this organism to drugs. Therefore, the purpose of this paper is to describe simple mediums for the growth of *H. gallinarum* and also a convenient method for its isolation in pure culture from infected chickens, as well as to record some of the growth and biochemical characteristics of this pathogen.

LITERATURE

Primary isolation of the fowl coryza bacillus was first reported in Europe by DeBlieck,[4,5] and in the United States by Nelson.[15] DeBlieck[4,5] obtained the best growth of his organism, which he named *Bacillus haemoglobinophilus coryza gallinarum*, on 25 per cent horse blood agar heated to 70 C. Nelson[15,16] isolated a hemophilic bacillus which he recovered from certain Berkefeld filtrates of nasal exudate inoculated in blood at the base of nutrient agar slants. The organisms grew in the blood at the bottom of the slant but failed to colonize on its exposed surface. They likewise failed to colonize on open blood agar plates, but colonization was obtained by sealing the plates with clay.

The next investigation on this subject was reported by Delaplane and co-workers,[6] who isolated a hemophilic bacillus from a very severe type of respiratory disease in Rhode Island in 1933. They considered as the best and most convenient medium nutrient agar slants to which sterile chicken blood (in 2% sodium citrate solution) has been added. This organism grew also on blood agar plates (blood from chicken, ox, horse, or pheasant) and bactohemoglobin plates, but not on blood-free mediums such as serum agar plates or in serum broth. These workers also stressed the importance of moisture as a highly contributing growth factor, and suggested that the organism be called by its present name *H. gallinarum*. This same name was proposed, independently and during the same year, by Eliot and Lewis.[10] The latter investigators tried to isolate and grow their organism on Noguchi Leptospira medium and then in chicken-embryo cultures in either plasma or Locke-Lewis solution, but toward the end they used 5 per cent blood agar. They mentioned that a partial atmosphere of carbon dioxide produces a marked stimulation of growth and a more regular morphology in young cultures; moreover, in this manner, optimum growth was obtained within the first twenty-four hours rather than at about forty-eight hours in normal atmosphere. During the same year Schalm and Beach[17] reported the isolation of a similar organism from California. According to this and their following studies,[18,19] this bacillus grew very well on boiled blood agar; however, it did not grow aerobically but only in an atmosphere of 10 per cent CO_2 or in sealed plates. All cultures required the presence of both the X and the V factors* for growth on an artificial medium.

While Delaplane *et al.*[7] obtained some scanty growth only in a medium consisting of small pieces of sterile raw potato in broth, Kessens[14] reported that the best liquid medium was 10 per cent blood broth containing a piece of raw potato. Delaplane and co-workers[8,9] continued their investigations of the growth requirements of *H. gallinarum* and discovered that hemophilic bacteria can grow very well on a blood-free medium by utilizing live, growing yeast to furnish the necessary growth factors. According to Gregory[11] "organisms failed to colonize on blood agar unless incubated in an atmosphere of increased CO_2 tension, and even

From the Government Poultry Field Laboratory, Livestock Department, Ministry of Agriculture, Tel Aviv, Israel.

Published with the approval of the director of the Livestock Department, Israel Ministry of Agriculture.

* The "X factor" is heat-stable, associated with hemoglobin, and replaceable by hematin, while the "V factor" is heat-labile, found in yeast, various vegetable extracts, as well as in whole blood.

then growth often fails." Blood broth and liver filtrate gave good results. Hofstad [12] used chicken infusion agar into which was incorporated 10 per cent chicken serum before slanting the agar, and the organisms colonized on the slants in a 10 per cent CO_2 atmosphere. Cunningham and Stuart [8] reported that "egg-yolk nutrient agar slants containing 0.8% sodium chloride with physiological saline at the base of the slants provide a satisfactory, easily prepared, and blood-free medium." The Russian worker [20] re-emphasized the importance of a moist environment in order to prevent the drying out of the medium, as originally reported by Delaplane *et al.*[6]

DeBlieck,[4] Nelson,[15] Schalm and Beach,[17] and Eliot and Lewis [10] isolated the organism from the nasal exudate, a tedious and difficult accomplishment. Delaplane *et al.*[7] isolated their bacillus from the nasal swelling: chickens showing edematous facial swellings were killed, the skin over the swollen area seared, and the seared skin incised with a hot instrument. A bacteriological loop was used to obtain some of the edematous fluid for inoculation of the medium. According to these workers, it was essential to attempt the isolation within twenty-four hours after the swellings were noticed, since at the 48-hour period results were often negative. A similar procedure was used by Beach and Schalm [1] for the weekly transfers of exudative material.

In connection with the maintenance of *H. gallinarum* in culture, Delaplane *et al.*[6] reported that transfers of the culture (in blood at the base of nutrient agar slants) every four to five days kept the organisms pathogenic for at least 40 transfers. Schalm and Beach [19] reported that transfers at intervals of less than fourteen days are necessary for the maintenance of slant or plate cultures, and stock cultures can be maintained indefinitely by making transplants once a week. Kessens [14] has pointed out that *H. gallinarum* does not survive for more than two weeks in culture tubes, and that this limitation differentiates it from *Hemophilus influenzae*, which survives for four weeks.

Materials and Methods

Solid Culture Medium.—After trying out blood agar slants composed of blood from various species and in amounts ranging from 5 to 25 per cent, we settled on slants containing 10 per cent sheep blood as most convenient for us, although not necessarily the best medium available for the culture of *H. gallinarum*. Undoubtedly, chicken blood is superior to sheep blood, especially for the primary isolation and until the culture has become adapted to growth on artificial medium; however, in a partial atmosphere of carbon dioxide, the latter yields satisfactory results. Chocolate-agar, supposedly the medium of choice for the genus Hemophilus, is rather difficult to use, because the very tiny colonies are hardly visible against such a dark background.

Liquid Culture Medium.—Although Delaplane *et al.*[6] mentioned that they obtained no growth in serum broth, in our experience plain nutrient broth to which has been added about 10 per cent chicken-blood serum makes a simple but very satisfactory liquid medium. However, it is essential that this serum should be from the blood of chickens because serum obtained from rabbits or cattle was ineffective and did not support growth. Another satisfactory liquid medium consists of nutrient broth containing 10 per cent citrated blood. The disadvantage of this blood-broth is the fact that it takes almost forty-eight hours for the blood to settle completely to the bottom of the tube, and until that time it is impossible to distinguish the turbidity caused by the growth of the organism from that caused by floating blood cells.

Carbon Dioxide Atmosphere.—Of the various methods tried to stimulate the growth of *H. gallinarum*, we consider the partial atmosphere of CO_2 (approximately 10%) as the most important and successful one. However, in contrast to the reports of Nelson,[15, 16] Schalm and Beach,[17, 18] or Gregory,[11] and in agreement with DeBlieck [5] and Delaplane *et al.*,[6] scanty growth and colonization were obtained on the surface of blood agar also under aerobic conditions. This CO_2 atmosphere reduced the advantage of chicken blood agar over regular blood agar. It was produced by pouring sulfuric acid on sodium carbonate inside a wide-mouthed tube, which together with culture tubes and plates had been placed into an air-tight container. Besides the release of carbon dioxide, this chemical reaction also produced water, the evaporation of which during incubation may have contributed to the good results, since Delaplane and co-workers [6] and Svintsov [20] stressed the importance of a moist environment as growth factor.

Culture Technique and Transfers.—Due to the diminutive size of the colonies of *H. gallinarum*, which usually can be seen only with the aid of a magnifying glass, any unevenness on the surface of the slants or plates makes their recognition rather difficult. This is especially true for the traces left by a bacteriological loop. Therefore, as much as possible, the organisms were kept in broth and transferred by means of pipettes. Every transfer was checked for sterility by simultaneous inoculations of nutrient agar and blood agar slants. In the former case, a few drops of broth were allowed to roll slowly down over the surface of the slant, which then was incubated aerobically. In the latter case more drops were used, and then the slant was inclined until its surface became horizontal in order to let the inoculum penetrate; incubation in CO_2 atmosphere followed. Such inoculation of blood agar slants caused a very fine confluent growth, so fine that individual colonies were not readily detected by the unaided eye. However, when growth was scant, the colonies were larger and more distinctly visible. When the need arose to transfer organisms from such blood agar slants, about 1 cc. of serum broth was added to the slant and after vigorous shaking was left to incubate for twenty-four hours; thereafter, transfers were made with a pipette as described previously.

Since cultures on solid or in liquid mediums rarely remained viable for more than two weeks, all strains were routinely transferred once a week. However, in some exceptional cases, the viability of cultures, which were well adapted to artificial medium, was not lost even after one month at 4 to 6 C.

Cultivation in Developing Egg Embryos.—At the suggestion of Dr. A. Komarov an attempt was made to grow *H. gallinarum* in embryonating eggs. Inoculation into the yolk sac of 7-day-old embryos caused 100 per cent mortality within twenty-four to forty-eight hours, the great majority of embryos dying within twenty-four hours, while inoculation into the allantoic sac of 10-day-old embryos caused a slower and rather irregular mortality. The dead embryos appeared very congested, and a heavy growth of the organism was obtained in both the yolk and the allantoic fluid. The organisms in these two fluids remained viable for six weeks in a household refrigerator, as compared to two weeks in the case of artificial medium. There can be no doubt that the developing chicken embryo is superior to all other mediums described, with regard to convenience, density of growth, and length of viability. However, since the sensitivity tests to be described in the next paper of this series had been started on artificial medium, and since they were performed during a season of relative scarcity of fertile eggs, they were continued on the medium described. Further work on the use of embryonating eggs for studies on *H. gallinarum* is in progress. In laboratories where chicken serum is not readily available, plain nutrient broth containing 10 per cent allantoic fluid makes a satisfactory liquid medium for *H. gallinarum*.

Isolation of the Organisms from Infected Chickens.—Isolations were attempted only from facial swellings and never from nasal exudate. The edematous area is swabbed well with cotton moistened with alcohol, the skin is pierced by a rotary movement with the pointed end of a Pasteur pipette, then the fluid content of the swelling is sucked into the pipette and discharged immediately onto a blood agar slant and into serum-broth. Often in the early stage of the edema the surrounding tissues are hyperemic, and a mixture of exudate and blood will be obtained. This has the disadvantage of requiring a speedy technique to prevent clotting of the blood in the thin end of the pipette but has the advantage of representing an excellent culture medium on which better and more easily visible colonization is obtained.

Determination of Biochemical Reactions.—In order to facilitate the growth of *H. gallinarum* in carbohydrate and other mediums, one or more drops of chicken serum were added in order to obtain a medium containing at least 5 per cent serum, and to this was added the inoculum consisting of 2 drops of a 48-hour broth-culture (containing 10% serum). All tests were incubated in a partial atmosphere of CO_2 and were read after forty-eight and ninety-six hours. The tubes with carbohydrate fermentation broth did not contain inverted vials, therefore the presence or absence of gas formation during fermentation could not be determined. In a number of these tubes enough acid formed due to the CO_2 in the atmosphere to induce a slight acid reaction of the indicator; however, this could be differentiated from bacterial acid formation by additional incubation under aerobic conditions, whereupon the former disappeared.

RESULTS AND DISCUSSION

Isolation of Hemophilus gallinarum from Chickens Suffering from Coryza.—It seems to the authors that their technique for the isolation of organisms responsible for infectious coryza in chickens is superior to the ones published up to now. It is simple, fast, can be used in the field, and does not necessitate the destruction of the chicken. The bird is not harmed, except for a temporary swelling at the site of the puncture and some watering of the eye due to the alcohol. At times it is necessary to stop the hemorrhage with cotton. Moreover, the above procedure can be used several times on the same chicken, in order to determine the action of therapeutic agents *in vivo*.

The only prerequisite is to attempt the isolation only from facial swellings of one to three days' duration, that is, from slight swellings devoid of tenseness. Delaplane *et al.*[7] also maintained that "the success of the isolation depends on the length of time the swellings have persisted." However, they discussed the viability of the pathogenic organism, while we are concerned with the presence of contaminating secondary invaders. These authors report failure to isolate the pathogenic bacillus in 5 of the 6 fowls examined forty-eight hours after noticing the swellings about the face. However, in our experience, *H. gallinarum* can be isolated from swellings of much longer duration, but they may be heavily contaminated. Due to the relatively scanty growth of *H. gallinarum* in contrast to the abundance of the contaminants, separation of the two is difficult and usually an unsuccessful task.

The following case may serve as an example of the efficiency of the procedure described above. In an *in vivo* experiment, the authors visited an infected flock of 4-month-old pullets and selected 6 having a facial edema in the suitable stage for isolation of *H. gallinarum*. These were transferred to the feed room, where the "labora-

tory'' consisted of a small bottle of alcohol, some cotton, a package of sterile Pasteur pipettes, a few blood agar slants, and a small alcohol burner (over the flame of

TABLE 1 — Geographical Distribution of the Flocks from Which the Various Strains of Hemophilus Gallinarum Were Isolated

Strains	Date of isolation	Air distance (miles) and direction from Tel Aviv
II, III	1/27/53	11 NE*
37, C, Y	6/ 4/53	10 NE*
46, 50, 65, 79	7/ 6/53	2½ SW**
101, 102	7/ 6/53	2½ SW**
123	7/ 9/53	2½ SW**
528	8/ 3/53	3 SE
793	8/21/53	13 S
917	8/30/53	10 SSE
1111	9/20/53	8 E
1124	9/21/53	2 SWW
1249	10/ 9/53	28 SE
1375	10/28/53	12 SE
1446‡	11/15/53	51 NNE
1473, 1474‡	11/19/53	68 NNE
1487†	11/24/53	15 SE
GI‡	12/10/53	51 NE

* Two farms on opposite ends of the same village.

** Three accessory farms in the same neighborhood but divided by streets and without fences in common.

† This flock became infected about one week after the introduction into the farm of 3-month-old pullets bought three to four miles south of Tel Aviv.

‡ These strains were isolated at the Poultry Field Laboratory, Haifa.

which the slants were opened during inoculation). Within thirteen minutes, and without harm to the birds, samples from the facial swellings had been collected. Of the six slants inoculated, five yielded pure cultures of *H. gallinarum* while one remained sterile.

Table 1 presents a survey of the geographical distribution of the various strains isolated and selected for further study. Rather than enumerate names of localities, an attempt is made to show this distribution on the basis of air-distances (as measured on a map) and directions from Tel Aviv, where this laboratory is located.

Cultural Characteristics.—During all of the attempts at culturing the edematous fluids of facial swellings of chickens suffering from apparently typical cases of infectious coryza, the only organisms which were isolated (besides obvious contaminants) were small gram-negative bacilli which did not grow on nutrient agar nor in plain broth, but which could be cultured relatively easily on or in medium with methods previously described.

Early in this bacteriological study, it became apparent that different strains had consistently different growth characteristics which were easily recognizable in 48-hour broth cultures. Strains C, Y, 46, 79, 101, 102, 123, 1111, 1446, and 1487 produced a light growth which caused only a slight clouding of the serum broth. Strains 37, 917, 1249, 1375, and GI produced a rather turbid broth with a flocculent sediment, while strains 528, 793, and 1473 were intermediate between the above two types, that is, they produced moderate clouding but no sediment. The significance of these growth characteristics is unknown, because they appear incidental and without association with etiologica' considerations or biochemical reactions.

Biochemical Reactions.—The literature on *H. gallinarum* contains few references to the biochemical reactions of this organism. It seemed worthwhile, therefore, to test the local strains as to their reactions in this respect, and to find whether differences would appear among the various strains isolated. The reactions tested were: indol and H_2S production, litmus and methylene blue milk reactions, gelatine liquefaction, and the fermentation of 20 carbohydrates.

None of the 11 strains tested produced indol or H_2S, neither did they act on litmus or methylene blue milks, nor did they liquefy gelatin. As far as the carbohydrates are concerned, all strains fermented glucose, but none fermented arabinose, rhamnose, xylose, lactose, trehalose, raffinose, inulin, dulcitol, mannitol, sorbitol, glycerol, inositol, and salicin. Strains 37, 917, and GI did not ferment any carbohydrate except glucose. The fermentation reactions of the other eight strains on six carbohydrates, in addition to glucose, were as follows: strain 46 fermented saccharose; strains 1111 and 1249 fermented maltose; strains C and 1473 each fermented three additional carbohydrates, mannose (slightly), dextrin, and saccharose or levulose; strains 102, 793, and 1375 fermented mannose (slightly), galactose, levulose, maltose, saccharose, and dextrin.

To the best of our knowledge, the only confirmation available in the literature for the above results are the reports of Delaplane *et al.*[6] who mentioned that indol is not produced, and of Eliot and Lewis,[10] who likewise stated that no indol is produced, but they found a definite acid for-

mation from dextrose. With regard to the biochemical reactions of other veterinary species of the genus Hemophilus, Watt [21] isolated an organism which slightly fermented glucose but none of the other nine carbohydrates tested, nor did it change litmus milk. Jackson [13] reported that his strains of *H. bovis* were negative to indol nor did they ferment carbohydrates, but gelatin was liquefied after six days at 22 C. and litmus milk turned alkaline after seven days.

The significance of the slight differences in the fermentation of six carbohydrates by eight of our strains is uncertain. Etiologically, strains 37 and C were isolated from the same flock, and strains 46 and 102 from two neighboring flocks; therefore, the variation in their biochemical behavior is difficult to understand, unless it be dismissed as accidental and unimportant.

It has been mentioned in a previous paragraph that the only organisms isolated were small gram-negative bacilli which did not grow on agar or in plain broth. Pure cultures of these organisms produced a slight but typical respiratory disease (with nasal discharge and facial swelling) in susceptible birds twenty-four to forty-eight hours after intranasal and intrasinal inoculations, and the same type of bacilli were re-isolated from these artificially infected birds, thus fulfilling Koch's postulates. On the basis of these facts, it is concluded that infectious coryza found in the chickens of this country is associated with *H. gallinarum*.

SUMMARY

The literature on the isolation and culture of *Hemophilus gallinarum* is reviewed, and simple solid and liquid artificial mediums for the culture of this organism, as well as a convenient procedure for its isolation (even under field conditions), are presented.

It is suggested that 7-day-old embryos, infected via their yolk sacs, support very well the growth of *H. gallinarum*.

This bacillus is rather inactive biochemically. It does not produce indol nor H_2S, does not act on litmus or methylene blue milks, and does not liquefy gelatin. Out of 20 carbohydrates tested it ferments consistently only glucose, and some strains also ferment one or more of the following six carbohydrates: mannose, galactose, levulose, maltose, saccharose, and dextrin.

It is concluded that *H. gallinarum* is involved in the etiology of avian infectious coryza in Israel.

References

[1] Beach, J. R., and Schalm, O. W.: Studies of the Clinical Manifestations and Transmissibility of Infectious Coryza of Chickens. Poult. Sci., *15*, (1936): 466-470.

[2] Bornstein, S., and Samberg, Y.: The Therapeutic Effect of Streptomycin on Infectious Coryza of Chickens Caused by *Hemophilus Gallinarum*. I. Blood Serum Levels of Streptomycin in Chickens. Am. J. Vet. Res., *15*, (1954): 607-611.

[3] Cunningham, C. H., and Stuart, H. O.: Egg Yolk Medium for the Cultivation of *Hemophilus Gallinarum* Am. J. Vet. Res., *5*, (1944): 142-146.

[4] DeBlieck, L.: En haemoglobinophile bacterie als oorzaak von coryza infectiosa gallinarum. Tijdschr. v. Diergeneesk., *58*, (1931): 310-318.

[5] DeBlieck, L.: A Haemoglobinophilic Bacterium as the Cause of a Contagious Catarrh of the Fowl. Vet. J., *88*, (1932): 9-13.

[6] Delaplane, J. P., Erwin, L. E., and Stuart, H. O.: A Hemophilic Bacillus as the Cause of an Infectious Rhinitis (Coryza) of Fowls. Rhode Island Agric. Exptl. Station Bull. 244, 1934.

[7] Delaplane, J. P., Erwin, L. E., and Stuart, H. O.: The Isolation of a Hemophilic Bacillus in Pure Culture and the Reaction of Chickens to Extranasal Inoculations Thereof. J. Agric. Res., *52*, (1936): 377-383.

[8] Delaplane, J. P., Erwin, L. E., and Stuart, H. O.: The Effect of the X Factor, of NaCl, and of the Composition of the Nutrient Media upon the Growth of the Fowl Coryza Bacillus, *Hemophilus Gallinarum*. J. Agric. Res., *56*, (1938): 916-926.

[9] Delaplane, J. P., and Stuart, H. O.: A Growing Yeast Medium for the Cultivation of Hemophilic Bacilli. J.A.V.M.A., *95*, (1939): 326-328.

[10] Eliot, C. P., and Lewis, M. R.: A Hemophilic Bacterium as a Cause of Infectious Coryza in the Fowl. J.A.V.M.A., *84*, (1934): 878-888.

[11] Gregory, D. W.: A Superior Medium for the Hemophilic Fowl Coryza Bacillus. Am. J. Vet. Res., *4*, (1943): 32-33.

[12] Hofstad, M. S.: Unpublished Data, 1943 (cited in "Diseases of Poultry," ed. by Biester and Schwarte, 3rd ed., Iowa State College Press, Ames [1952]: 378).

[13] Jackson, F. C.: Infectious Keratoconjunctivitis of Cattle. Am. J. Vet. Res., *14*, (1953): 19-25.

[14] Kessens, B. H.: Comparison Between Hemophilus Coryza and Hemophilus Influenzae. Vet. Bul., *9*, (1939): 452 (abstr. thesis, 1936).

[15] Nelson, J. B.: Etiology of an Uncomplicated Coryza in the Domestic Fowl. Proc. Soc. Exptl. Biol. Med., *30*, (1932): 306-307.

[16] Nelson, J. B.: Studies of an Uncomplicated Coryza in the Domestic Fowl. I. Isolation of a Bacillus which Produces a Nasal Discharge. J. Exptl. Med., *58*, (1933): 289-296.

[17] Schalm, O. W., and Beach, J. R.: The Etiology of a Respiratory Disease of Chickens. Science, *79*, (1934): 416-417.

[18] Schalm, O. W., and Beach, J. R.: Cultural Requirements of the Fowl-Coryza Bacillus. J. Bact., *31*, (1936): 161-169.

[19] Schalm, O. W., and Beach, J. R.: Studies of Infectious Coryza of Chickens with Special Reference to its Etiology. Poult. Sci., *15*, (1936): 473-481.

[20] Svintsov, P. M.: Infectious Coryza in Fowls (in Russian). Veterinariya, *27*, (1950): 31-37.

[21] Watt, J. A. A.: The Isolation and Cultural Characteristics of an Organism of the Haemophilus Group in Calf Pneumonia. J. Comp. Path., *62*, (1952): 102-108.

Corynebacterium Pyogenes—A Cultural and Serological Study

J. F. RYFF, D.V.M., and JO BROWNE, D.V.M.

Laramie, Wyoming

Corynebacterium pyogenes is a common pathogen which is found in pyogenic processes in various species of animals. Among the specimens examined for diagnostic purposes in this laboratory, *C. pyogenes* was more frequently isolated than any other pathogenic organism although in many cases it could be considered a secondary invader.

Corynebacterium pyogenes is generally accepted as a separate species. Bergey[1] characterized the species as a gram-positive, pleomorphic rod which produces light growth on artificial mediums, produces slight liquefaction of serum mediums, causes *beta* hemolysis and coagulation of milk. All cultures are reported to ferment dextrose and usually lactose, maltose, and sucrose; variable reactions are observed with arabinose, dextrin, glycerol, mannitol, salicin, and xylose; in a few instances fructose, galactose, and levulose have been fermented but not dulcitol, inulin, raffinose, rhamnose, sorbose, or sorbitol.[1, 2-9] Morse[9] considered *C. pyogenes* the most active in carbohydrate fermentation and the only species which causes *beta* hemolysis. Brooks and Hucker[10] suggested that the corynebacteria organisms be divided into three groups based on the fermentation reactions: an active group represented by *Corynebacterium equi*, an intermediate group represented by *Corynebacterium flavidum*, and an inactive group represented by *C. pyogenes*.

A toxin has been described but the use of toxoid has not protected cattle against summer mastitis due to *C. pyogenes*.[8, 11-14] Brown and Orcutt,[4] using the precipitation test, found no difference between twelve strains. Minett,[5] Jowett,[6] and Merchant[7] reported serological studies of various strains of *C. pyogenes* which suggested antigenic similarity. Lovell[8] concluded from a study of five strains that there is a major and a minor antigen which can be demonstrated serologically.

Materials and Methods

Fifty-seven cultures of *C. pyogenes* were employed. As soon as they were adapted to artificial medium by continued subculturing, they were maintained by monthly or bimonthly transfers and were refrigerated in the interim. For fermentation studies, Difco's phenol red broth base, with 0.5 per cent of the desired agent, was inoculated and observed for three weeks at 37 C. For the catalase test, it was found necessary to place hydrogen peroxide on a 24-hour agar slant.[15] Even then, gas bubbles were apparent only after twenty to thirty minutes with some strains. For serological procedures, cultures were grown in nutrient broth containing 1.0 per cent tryptose and 1.0 per cent dextrose and, after centrifugation, the packed cells were washed twice with 0.85 per cent saline. In investigations of the somatic makeup, the washed cells were resuspended in saline to one tenth of the original broth volume and heated in flowing steam for one hour. This was injected intravenously into rabbits in 2- to 3-cc. amounts at three-day intervals approximately twelve times. The rabbits were then bled ten days after the last injection. While these rabbit serums were not

TABLE 1—Sources and Association of 57 Strains of Corynebacterium Pyogenes in Eight Species

Organisms or conditions associated with	Number of strains								
	Calf	Cattle	Sheep	Hog	Dog	Antelope fawn	Deer	Chicken	Pigeon
Pasteurella	4	1							
Paracolon organisms and *beta* streptococci	1								
Salmonella and *beta* streptococci	1								
Clostridium chauvoei (feseri)	2								
Clostridium septicum	1								
Actinomyces boris		1							
Spherophorus necrophorus				1					
Bacteremia*	3	7	1	2					
From brain		1	1						
From lungs							3		
Respiratory infection only bacterial pathogen	6		1						
Respiratory infection with *beta* streptococci	2								
Facial cellulitis	2								
Nephritis from sulfonamide therapy	1								
Hyperkeratosis	1								
Liver abscess		3							
"Brisket disease syndrome"		1							
Pulmonary emphysema		1							
Sinusitis			1						
Conjunctivitis			1						
Arthritis			1						
Encephalitis					1				
Thallium poisoning					2				
Hepatitis						1			
Newcastle disease and pullorum disease								1	
Rickets									1
Total	24	15	6	3	3	1	3	1	1

* Recovery from tissues submitted. This may not represent true bacteremia.

From the Wyoming State Veterinary Laboratory, Laramie. Dr. Jo Browne is now with Washington State Department of Agriculture, Puyallup.

checked individually for the absence of antibodies against *C. pyogenes* prior to immunization, two sets of three rabbit serums were each pooled and tested against the ten selected bacterial strains

TABLE 2—Results Obtained in the Fermentation Tests of 57 Strains of Corynebacterium Pyogenes

Carbohydrate	No. of strains producing acid: No. of strains	at (days)	No. of strains	at (days)	No. strains not tested
Aesculin	54	1 to 7	1	7 to 21	1
Arabinose	28	1 to 7	6	7 to 21	2
Dextrin	56	1 to 7	0	7 to 21	
Dextrose	56	1 to 7	0	7 to 21	
Dulcitol	0	1 to 7	0	7 to 21	
Galactose	56	1 to 7	1	7 to 21	
Glycerol	29	1 to 7	16	7 to 21	
Inositol	8	1 to 7	2	7 to 21	1
Inulin	9	1 to 7	1	7 to 21	
Lactose	54	1 to 7	1	7 to 21	
Levulose	54	1 to 7	2	7 to 21	
Maltose	56	1 to 7	0	7 to 21	
Mannitol	35	1 to 8	2	7 to 21	
Raffinose	25	1 to 7	10	7 to 21	
Rhamnose	24	1 to 7	3	7 to 21	
Salicin	52	1 to 7	3	7 to 21	
d-Sorbitol	28	1 to 7	1	7 to 21	1
Starch	53	1 to 7	0	7 to 21	
Sucrose	46	1 to 7	7	7 to 21	
Trehalose	55	1 to 7	0	7 to 21	1
d-Xylose	38	1 to 7	9	7 to 21	1
l-Xylose	33	1 to 7	9	7 to 21	1

without agglutination at 1:25 or above. The same general procedure was employed in investigating the capsular antigen, except that the supernatant from the original eighteen-hour broth culture, treated with sufficient formalin to make 0.5 per cent and held at 37 C. for forty-eight hours, was used as the inoculum.

For the agglutination test, the antigen was the same broth-grown, washed cells, untreated with disinfectants, and freshly harvested. All suspensions were adjusted to the same standard turbidity (50-60 % transmission against distilled water blank, red filter 650, Lumetron photoelectric colorimeter). Doubling dilutions of serum were made from 1:50 and agglutination read after incubation at 37 C. for twenty-four hours. In agglutinin absorption, a 1:10 dilution of serum in saline was held in contact overnight at 37 C. with an equal volume of heavy bacterial suspension. After centrifugation, the supernatant was utilized in appropriate dilutions for agglutination tests.

Use of formolized bacterial suspensions in 5-cc. amounts required a greater number of injections into rabbits and even then high titers were not consistently secured. The agglutination test with these serums was first set up after the antiserums were absorbed with a suspension of the homologous culture that had been heated in flowing steam. The results were so similar to that obtained by employing the fresh, washed cells without absorption that this step was abandoned. Antiserums against formolized cultures produced an agglutination test in which the end point was difficult to determine. The titer was interpreted as the last tube with pronounced agglutination, and this may explain the low titers reported.

The cultures used to inoculate rabbits and for subsequent agglutination studies appeared to be smooth forms.

RESULTS

As many of the strains of *C. pyogenes* used in this study were recovered from organs or tissues in which a pathological diagnosis could not be made, a classification of their connection with disease processes is not entirely satisfactory, but the sources of the fifty-seven strains are listed in table 1.

Following primary isolation of the cultures, it was not unusual to find small cocci, sometimes in short chains. Later they appeared uniformly as short, plump rods with occasional spherical forms. Subculturing on blood agar or Löffler's medium did not bring about a return to the original morphology. All were hemolytic on sheep blood

TABLE 3—Agglutination of Corynebacterium Pyogenes with Antiserums Prepared Against Heated Cultures

Antiserum against heated culture (No.)	Strain (No.): 1945–9	4629–3	5060–1	5521–5	7127–1	7302–1	7451–4	13178–4	13212–1	14954–5	Source of culture
1945–9	*3200*	1600	50	0	0	1600	1600	0	0	50	Ovine conjunctivitis
4629–3	800	*800*	200	100	100	400	400	50	0	100	Antelope hepatitis
5060–1	50	100	*400*	400	100	50	200	200	0	0	Ovine pneumonia
5521–5	200	200	400	*400*	50	100	200	0	200	100	Bovine emphysema
7127–1	200	200	400	200	*800*	200	800	0	0	100	Swine bacteremia
7302–1	200	400	100	200	200	*400*	400	0	50	50	Calf pneumonia
7451–4	1600	800	50	50	0	800	*1600*	0	200	100	Calf pneumonia
13178–4	0	0	0	0	0	0	0	*400*	0	100	Calf pneumonia
13212–1	0	50	0	0	0	50	50	200	*400*	400	Calf pneumonia
14954–5	50	50	100	0	100	50	100	0	100	*800*	Calf with pasteurellosis

0—no agglutination at 1:50; italics—homologous titer; others have end point of agglutination expressed as reciprocal of highest dilution showing pronounced agglutination.

TABLE 4—Agglutination Titer Against Homologous Strain After Absorption with Various Strains

Antiserum	Strain used for absorption									
	1945–9	4629–3	5060–1	5521–5	7127–1	7302–1	7451–4	13178–4	13212–1	14954–5
1945–9	0	0	200	0	0	50	200	200	200	200
4629–3	100	0	50	100	0	100	100	100	100	400
5060–1	0	0	0	0	0	50	0	400	100	0
5521–5	0	50	200	0	0	100	200	100	100	200
7127–1	200	0	100	50	0	0	50	100	100	200
7302–1	50	200	50	400	0	0	50	200	100	200
7451–4	100	200	50	100	50	50	0	200	0	100
13178–4	100	0	100	0	0	100	100	0	100	100
13212–1	0	100	0	400	0	50	50	100	0	50
14954–5	200	0	X	0	0	50	X	X	X	0

0—no agglutination at 1:50; X—not tested; homologous titers given in table 3.

agar when isolated. Usually the initial light growth became heavier with transferring and, when returned to blood agar after an interval of up to four years, their hemolytic properties were decreased or lost. A few cultures that failed to make this adjustment to stock culture medium were carried on blood agar and remained hemolytic. After being carried in this fashion, one of the 57 strains liquefied Löffler's medium, although during this adaptive process three others were still capable of liquefaction.

With litmus milk, three strains had no effect; two produced acid; 51 formed a soft or acid curd; and one produced early peptonization. Thirty strains produced reduction of litmus; eight caused a marked separation of whey; and 11 progressed to peptonization. Thirty one of the 57 strains liquefied gelatin. Forty seven strains produced detectable hydrogen sulfide on Difco lead acetate agar. With Difco nitrate agar, nine strains at some time or other reduced nitrate to nitrite, although eight of these also had several negative tests. In 1 per cent tryptone broth, none of the strains produced indol. Two strains alone hydrolyzed sodium hippurate. All strains produced catalase.

The results obtained in the fermentation tests are summarized in table 2.

Ten strains randomly selected, except to include isolates from different species of animals, were used in the investigation of somatic structure. The titers of the antiserums against their homologous culture and the other nine strains are given in table 3.

Examination of the cross reactions indicates a close relationship in somatic antigenic structure among these ten strains except for the weak relationship shown for cultures 13178–4 and 13212–1.

To further demonstrate the relationship existing between these strains of *C. pyogenes*, agglutinin absorption was utilized. For this, the same antiserums as described in table 3 were employed. Referring to line 1 of table 4, when antiserum against 1945-9 was absorbed with strain 1945-9 and tested with strain 1945–9, agglutination could not be detected at 1:50 dilution. In the same way, strains 4629–3, 5521–5, and 7127–1 were able to absorb all of the agglutinins from antiserum 1945–9 so that it was no longer capable of agglutinating strain 1945–9. Agglutinin absorption verifies the close relationship between 1945–9 and

TABLE 5—Heterologous Agglutination Following Absorption

Antigen	14954–5 antiserum absorbed with 1945–9				5060–1 antiserum absorbed with 13178–4				7302–1 antiserum absorbed with 5521–5			
	1/50	1/100	1/200	1/400	1/50	1/100	1/200	1/400	1/50	1/100	1/200	1/400
1945–9	—	—	—	—	—	—	—	—	+	+	—	—
4629–3	—	—	—	—	—	—	—	—	+	+	+	+
5060–1	—	—	—	—	+	+	+	+	+	+	+	—
5521–5	—	—	—	—	—	—	—	—	—	—	—	—
7127–1	+	+	+	+	+	+	+	+	+	+	+	+
7302–1	—	—	—	—	—	—	—	—	+	+	+	+
7451–4	+	I	—	—	—	—	—	—	+	+	I	—
13178–4	—	—	—	—	—	—	—	—	—	—	—	—
13212–1	+	+	+	+	+	+	+	+	+	+	+	+
14954–4	+	+	+	I	+	+	+	+	+	+	+	+

+—definite agglutination; I—incomplete agglutination.

TABLE 6—Agglutination of Corynebacterium Pyogenes with Antiserums Prepared Against Formolized Cultures

Antiserum against formolized cultures (No.)	Strain										
	1945–9	4029–1	4629–3	5060–1	5521–5	7127–1	7302–1	7451–4	13178–4	13212–1	14954–5
1945–9	*1600*	X	800	800	50	200	800	1600	200	50	0
4029–1	400	*400*	200	200	50	200	200	200	200	200	200
4629–3	400	X	*400*	100	100	200	400	100	200	200	200
5060–1	400	X	200	*1600*	400	400	400	400	200	200	200
5521–5	100	X	100	100	*200*	200	100	100	200	100	50
7127–1	100	X	50	100	100	*200*	100	100	100	100	100
7302–1	200	X	100	100	200	200	*400*	200	100	100	200
7451–4	200	X	200	400	100	100	400	*800*	50	50	50
13178–4	200	X	50	200	200	200	200	50	*400*	200	200
13212–1	50	X	0	50	200	200	100	100	100	*400*	200

X—not tested; 0—no agglutination at 1:50; end point of agglutination expressed as reciprocal of highest dilution showing pronounced agglutination. Italics—homologous titer.

4629–3 and 7302–1 but indicates their somatic makeup is not identical. Strains 1945–9 and 5521–5, 4629–3 and 7127–1, and 7127–1 and 7302–1 were capable of complete cross absorption, yet some difference apparently exists because 4629–3 and 7302–1 can not be so directly compared. Simple cross agglutination (table 3) also suggests that these pairs are not antigenically identical. The marked reduction in titers seen with agglutinin absorption, like the cross-agglutination procedure, would indicate possession of a fairly close relationship between strains.

To carry this absorption further, a few trials were made to determine the heterologous titers following heterologous absorption, and these results are given in table 5. These would suggest a common factor to several of these strains but also indicates that many strains are possessed of minor differences.

When 14954–5 antiserum with a homologous titer of 1:800 was brought into contact with 42 strains exclusive of those in table 3, 4 agglutinated to 1:800; 4, 1:400; 6, 1:200; 12, 1:100; 10, 1:50, and 6 showed no agglutination at 1:50. Thirteen of such strains set up against 7127–1 antiserum with a homologous titer of 1:800 resulted in agglutination to 1:200 with 2, 1:100 with 5, 1:50 with 5, and no agglutination with 1 at 1:50. Used with 30 strains, antiserum 7451–4, homologous titer 1:1600, produced agglutination to 1:400 with 3, 1:200 with 6, 1:100 with 8, 1:50 with 8, and no agglutination at 1:50 with 5. With 4 strains, antiserum 13212–1, homologous titer 1:400, affected agglutination to 1:400 with 1, 1:50 with 1, and no agglutination at 1:50 with 2. This would again indicate possession of somatic antigenic properties common to many strains.

These previous results were all based on antiserums prepared against the somatic fraction. In addition, antiserums were prepared against formolized antigens which would emphasize the capsular antigenic factor. In table 6 an even closer relationship between the different strains is shown when an antiserum is tested with heterologous strains. As preliminary work suggested immunity to live cultures was dependent upon the somatic antigen, the procedures with the antiserums against the capsular fraction were not carried beyond the cross-agglutination stage.

SUMMARY

After adaptation to unenriched mediums, *Corynebacterium pyogenes* cultures may be expected to retain their typical effect on litmus milk but lose their ability to liquefy Löffler's medium and, to some extent, their hemolytic properties and ability to liquefy gelatin. Such adaptation enhances their carbohydrate fermentation properties and of 57 strains, 50 or more produced acid from aesculin, dextrin, dextrose, galactose, lactose, levulose, maltose, salicin, starch, sucrose, and trehalose; at least half fermented arabinose, glycerol, mannitol, raffinose, sorbitol, and xylose. Duleitol alone was not broken down by any of the strains. Cross agglutination and agglutinin absorption carried out with antiserums prepared against the somatic component of ten strains indicated a consistent relationship within the species. Such antiserums employed against other of the 57 strains also

indicated a relationship. Cross agglutination employing ten antiserums prepared against the capsular fraction indicated an even closer relationship between strains.

References

[1] Breed, R. S., Murray, E. G. D., Hitchens, A. P.: Bergey's Manual of Determinative Bacteriology. 6th ed. Williams and Wilkins Co., Baltimore, (1948): 381-382, 388.

[2] Smith, Louis DS.: A Key for Tentative Recognition of Bacteria Commonly Encountered in Clinical Bacteriology. Am. J. Vet. Res., *13*, (1952): 430-432.

[3] Wilson, G. S., Miles, A. A.: Principles of Bacteriology and Immunity. 3rd ed. Williams and Wilkins Co., Baltimore, *1*, 1946.

[4] Brown, J. Howard, and Orcutt, Marion L.: A Study of *Bacillus Pyogenes*. J. Exptl. Med., *32*, (1920): 219-248.

[5] Minett, C. F.: Diphtheroid Bacilli from Animals. J. Comp. Path. and Therap., *35*, (1922): 291-302.

[6] Jowett, Walter: *Bacillus Pyogenes* and Its Relation to Bovine Mastitis. J. Comp. Path. and Therap., *38*, (1935): 180-204.

[7] Merchant, I. A.: A Study of the Corynebacteria Associated with Diseases of Domestic Animals. J. Bact., *30*, (1935): 95-118.

[8] Lovell, Reginald: Studies on *Corynebacterium Pyogenes* with Special Reference to Toxin Production. J. Path. and Bact., *45*, (1947): 339-355.

[9] Morse, Erskine V.: Criteria for the Identification of Corynebacteria Isolated from Animals. Cornell Vet., *39*, (1949): 266-276.

[10] Brooks, R. F., and Hucker, G. J.: A Study of Certain Members of the Genus Corynebacterium. J. Bact., *48*, (1944): 295-312.

[11] Lovell, Reginald: Further Studies on the Toxin of *Corynebacterium Pyogenes*. J. Path. and Bact., *56*, (1944): 525-529.

[12] Brown, James A., and Stuart, James D.: The Treatment of Summer Mastitis Using *Corynebacterium Pyogenes* Toxoid. Vet. Rec., *55*, (1943): 315-316.

[13] Weitz, Bernard: *Corynebacterium Pyogenes* Infection in Cattle with Special Reference to Summer Mastitis. Vet. Rec., *61*, (1949): 123-126.

[14] Lovell, R., Foggie, A., and Pearson, J. K. L.: Field Trials with *Corynebacterium Pyogenes* Alum-Precipitated Toxoid. J. Comp. Path. and Therap., *60*, (1950): 225-229.

[15] Simmons, James Stevens, and Gentzkow, Cleon J.: Laboratory Methods of the United States Army. 5th ed. Lea and Febiger, Philadelphia, 1944.

Summary of Toxicological Information on 2,4-D and 2,4,5-T Type Herbicides and an Evaluation of the Hazards to Livestock Associated with Their Use

V. K. ROWE, M.S., and T. A. HYMAS, D.V.M.

Midland, Michigan

THE USE OF herbicides for the control of undesirable vegetation has been practiced for many years. Some of the older materials commonly used are chlorates, arsenicals, compounds of boron, certain oils, and even table salt. While many of these materials are still being used for this purpose, research over the past decade has revealed a family of new herbicidal materials. They are the chlorinated phenoxyacetic acids and closely associated compounds of which 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) are prototypes.

Early in the development of these new herbicides, studies were undertaken by The Dow Chemical Company to determine their toxicity to warm-blooded animals. It is the purpose of this paper to make the acquired toxicological information available to others.

LITERATURE REVIEW

Bucher[1] in 1946 was among the first to report the results of experiments with small animals using 2,4-D. Temporary myotonia lasting from eight to twenty-four hours or more following a single injection of 150 to 250 mg./kg. was observed in mice, rats, rabbits, and dogs. Repeated injections of smaller amounts, 50 to 100 mg./kg./day to mice for ninety days, failed to elicit either a characteristic chronic syndrome or a striking histological picture. Mice undergoing this treatment became pregnant and bore apparently normal litters. Repeated injections of 2,4-D did not alter the rate of growth of two transplantable mouse sarcomas.

In 1947, Hill and Carlisle[2] published the results of toxicological studies involving various preparations of 2,4-D. In acute oral studies, they found the $l.d._{50}$ for mice to be 375 mg./kg.; for rats, 666 mg./kg.; for rabbits, 800 mg./kg.; and for guinea pigs, 1,000 mg./kg. The largest dose administered to monkeys without serious after-effects was 214 mg./kg.; 428 mg./kg. caused nausea, vomiting, lethargy, muscle incoordination, and head drop. These workers observed that all species reacted similarly and that there was no significant difference in potency between crude and purified preparations, or between the sodium or ammonium salts. Deaths from large doses were believed due to ventricular fibrillation; if death was delayed, myotonia, stiffness of extremities, ataxia, paralysis, and coma were observed.

In subacute studies, the same investigators observed intoxication in dogs after six daily intravenous injections of 25 Gm./kg.; rats were fed a diet containing 1,000 parts per million (p.p.m.) of 2,4-D for a month without harmful effects; guinea pigs tolerated ten doses of 100 mg./kg. over a twelve-day period; and the inhalation of the sodium salt as a dust failed to cause systemic effects in guinea pigs. The main evidences of chronic intoxication observed in rats were visceral congestion and edematous kidneys with degenerative changes in the tubules; only dogs exhibited hepatic damage with central degeneration and congestion.

In 1948, Bjorn and Northen[3] reported the results of studies conducted on chicks with an alkanolamine salt of 2,4-D. The acute oral lethal range was found to be 380 to 765 mg./kg. When given repeatedly (12 doses in 28 days), 28 mg./kg./dose was without effect, while 280 mg./kg./dose caused depression of growth. These authors express no concern about the likelihood of toxic effects in chickens under ordinary conditions of use and point out that at a spraying rate of 1 lb. of 2,4-D per acre (a normal rate of application) a chicken weighing 1 kg. would have to consume all the 2,4-D applied on 72 sq. ft. within a day or two to obtain a lethal dose.

In 1953, Drill and Hiratzka[4] reported the results of acute and chronic oral toxicity studies on 2,4-D and 2,4,5-T with dogs. These men found that the acute oral $l.d._{50}$ values were about 100 mg./kg. for each of the materials. At this dosage level, 2,4-D produced definite myotonia accompanied by anorexia and weight loss while 2,4,5-T produced only signs of mild spasticity. Both 2,4-D and 2,4,5-T were fed five times a week for ninety days at dosage levels of 2, 5, and 10 mg./kg. without adverse effects. At a dosage level of 20 mg./kg., both materials caused serious effects; 3 of 4 dogs on the 2,4-D died while all of the 4 dogs on 2,4,5-T died. The animals receiving the 2,4-D exhibited stiffness of the hind legs, difficulty in swallowing, bleeding of the gums, necrotic changes in the buccal mucosa, and mild liver and kidney changes. A significant decrease in the number of blood lymphocytes was observed terminally in 3 of the 4 animals. The toxic symptoms noted in the animals

From the Biochemical Research Department (Rowe) and the Agricultural Chemical Research Laboratory (Hymas), The Dow Chemical Co., Midland, Mich.

receiving 2,4,5-T were slight muscle spasm, and difficulty in swallowing. Histological studies revealed mild changes in the liver and kidneys.

The first experimental toxicological work conducted on farm animals was that of Mitchell and

TABLE 1—Materials Studied*

Common name	Chemical name
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
MCP	2-Methyl-4-chlorophenoxyacetic acid or 4-Chloro-o-toloxyacetic acid
silvex	2-(2,4,5-Trichlorophenoxy)-propionic acid

* These are basic chemicals used in herbicidal formulations.

co-workers.[5] These investigators pastured sheep and cows on foliage sprayed with more than recommended amounts of 2,4-D without effect. They also fed a lactating cow 5.5 Gm. of 2,4-D daily for 106 days without ill effects. Milk from the cow did not contain a demonstrable quantity of 2,4-D. No effects were observed in the calf to which this milk was fed, nor was there any 2,4-D found in the serum of the calf. The serum of the cow, however, contained 8.4 p.p.m. of 2,4-D though none was found in the liver, kidney, or fatty tissues.

Early in 1950, Grigsby and Farwell* published the results of experiments with 2,4-D and 2,4,5-T involving domestic livestock. In this study, alfalfa was sprayed with various herbicidal formulations including three different 2,4-D preparations and one 2,4,5-T preparation. Livestock including horses, dairy and beef cattle, sheep, swine, and chickens were immediately pastured in the freshly treated areas. In summary of this work, the authors stated, "Results of this experiment indicate that none of the herbicides used had any serious physiological effect upon the livestock involved. They also indicate that none of the livestock preferred any of the sprayed areas to those that were unsprayed; however, with the lots sprayed with 2,4-D herbicides, the livestock grazed the areas sprayed almost as well as the unsprayed. Since the rates used were two to four times greater than recommended dosage, it seems that the farm use of these materials for pasture weed control is a reasonably safe procedure."

Materials Studied

Commercial herbicides generally can be described as formulations of active ingredients. The active ingredient usually appears as a salt which is soluble in water or as an ester which is soluble in oil. Formulations may be designed for use as water solutions, water emulsions, oil solutions, oil emulsions with water, or dusts, depending upon the use for which they are intended. Basically, most formulations contain, besides the active ingredients, a dispersant, a solvent, a wetting agent, and perhaps a diluent. These are usually considered to be inert ingredients although they may have a profound influence upon herbicidal effectiveness.

In the studies reported herein, toxicological data are reported not only for the basic active ingredients and their chemical derivatives (table 1) but also for the finished formulations as they are marketed * (table 2).

Oral Administration of Single Doses

Procedure.—The toxicity of the various herbicidal materials and formulations when administered in single oral doses to various species of animals has been determined. All rats, guinea pigs, and rabbits used were young adult animals from the stock colonies of this laboratory. The mice were young adults obtained from Carworth Farms. The chicks used were New Hampshire Reds obtained from a commercial hatchery when about 3 days of age and were about 3 weeks of age when treated.

The test materials were administered by intubation unless otherwise indicated. Aqueous solutions were used wherever possible. Olive oil or corn oil solutions were used where water was not appropriate. Surviving animals were observed until recovery was certain, usually about two weeks.

Results.—The results of the acute oral studies are summarized in tables 3 and 4.

* The trade-named drugs listed in table 2 and in the text are products of The Dow Chemical Co., Midland, Mich.

TABLE 2—Herbicidal Formulations Studied

Trade name	Active ingredients (%)
2,4-Dow weed killer (formula 40)	65.0 Alkanolamine salts of 2,4-D
Esteron 44	44.0 Isopropyl ester of 2,4-D
Esteron 245 (old formulation)	33.3 Isopropyl ester of 2,4,5-T; 12.1 Mixed amyl esters of 2,4,5-T
Esteron 245 (present formulation)	65.3 Mono-, di-, tripropylene glycol butyl ether esters of 2,4,5-T
Esteron ten-ten	70.5 Mono-, di-, tripropylene glycol butyl ether esters of 2,4-D
Brush killer 50-50	27.2 Butyl esters of 2,4-D; 26.5 Butyl esters of 2,4,5-T
Brush killer T	52.2 Butyl esters of 2,4,5-T
Esteron 76 (used in oil solution only)	37.1 Isopropyl esters of 2,4-D; 39.0 n-Butyl esters of 2,4-D
Esteron 76E (used in either oil solution or water emulsion)	36.8 Isopropyl esters of 2,4-D; 38.8 Butyl esters of 2,4-D
Esteron brush killer (old formulation)	25.6 Isopropyl esters of 2,4-D; 24.4 Isopropyl esters of 2,4,5-T
Esteron brush killer (present formulation)	34.8 Mono-, di-, tripropylene glycol butyl ether esters of 2,4-D 33.0 Mono-, di-, tripropylene glycol butyl ether esters of 2,4,5-T
Kuron (was called H-1078)	64.5 Mono-, di-, tripropylene glycol butyl ether esters of silvex
Dow MCP amine weed killer	69.1 Alkanolamine salts of MCP

In all cases where the data were sufficiently extensive to allow statistical analysis, the $l.d._{50}$ values with their 19/20 confidence limits were determined by the method of Litchfield and Wilcoxon.[7] In all other cases, the $l.d._{50}$ values were estimated and the range between the dose allowing survival of all animals treated and the dose causing all animals to die is given. For comparative purposes, some of the val-

TABLE 3—Summary of Acute Oral Toxicity of Various Basic Herbicidal Materials

Material	Species	Sex	Vehicle	$l.d._{50}$ (19/20 confidence limits) (mg./kg.)
2,4-D (2,4-Dichlorophenoxyacetic acid)	Rats	M	Olive oil	375 (302-465)
	Mice	M	Olive oil	368 (312-434)
	Guinea pigs	M and F	Olive oil	469 (397-553)
	Chicks	M and F	Olive oil	541 (358-817)
	Dogs (4)		Capsule	Range 100 (25-250)
2,4-D, alkanolamine salts	Chicks (3)		Water (dosage on acid equivalent basis)	Range ..... (380-765)
2,4-D, sodium salt	Rats	F	Water	805 (610-1,063)
	Rats (2)		Water	666 .....
	Guinea pigs	M	Water	551 (417-727)
	Guinea pigs (2)		Water	1,000 .....
	Mice (2)		Water	375 .....
	Rabbits (2)		Water	800 .....
2,4-D, isopropyl ester	Rats	M and F	Olive oil	700 (569-861)
	Guinea pigs	M	Olive oil	550 (451-671)
	Mice	M	Olive oil	541 (398-736)
	Chicks	M and F	Olive oil	1,420 (1,127-1,789)
2,4-D, mixed butyl esters	Rats	F	Corn oil	620 (320-954)
	Guinea pigs	F	Corn oil	848 (604-1,190)
	Rabbits	F	Corn oil	424 (252-712)
	Mice	F	Corn oil	Range 713 (500-1,000)
	Chicks	M and F	Undiluted	2,000 (1,350-2,960)
2,4-D, mono-, di-, tripropylene glycol butyl ether esters	Rats	F	Corn oil	570 (510-640)
2,4,5-T (2,4,5-Trichlorophenoxyacetic acid)	Rats	M	Olive oil	500 (391-640)
	Mice	M	Olive oil	389 (245-619)
	Guinea pigs	M and F	Olive oil	381 (307-472)
	Chicks	M and F	Olive oil	310 (211-456)
	Dogs (4)		Capsule	Range 100 (50-250)
2,4,5-T, isopropyl ester	Rats	M and F	Olive oil	495 (420-584)
	Guinea pigs	F	Olive oil	449 (362-557)
	Mice	F	Olive oil	551 (380-799)
2,4,5-T, mixed butyl esters	Rat	F	Corn oil	481 (313-739)
	Rabbit	M	Corn oil	Range 712 (500-1,000)
	Mice	F	Corn oil	940 (674-1,312)
	Guinea pigs	F	Corn oil	Range 750 (500-1,000)
2,4,5-T, mixed amyl esters	Rats	F	Olive oil	Range 750 (500-1,000)
MCP (4-chloro-o-toloxyacetic acid or 2-Methyl-4-chlorophenoxyacetic acid)	Rat	M	Corn oil	Range 700 (500-1,000)
MCP, amine salt	Rat	M	Water	Range 1,200 (1,000-2,000)
	Guinea pigs	M	Water	Range 1,200 (630-2,000)
silvex, (2[2,4,5-trichlorophenoxy] propionic acid)	Rat	M and F	Corn oil	Range 650 (560-760)
silvex, mixed butyl esters	Rat	F	Corn oil	Range 600 (250-1,000)
	Rabbits	F	Undiluted	Range 750 (500-1,000)
	Chicks	M and F	Corn oil	1,190 (707-2,000)
silvex, mono-, di-, tripropylene glycol butyl ether esters	Rat	F	Corn oil	621 (473-814)
	Guinea pigs	M	Corn oil	Range 1,250 (500-2,000)
	Mice	F	Corn oil	Range 1,410 (1,000-2,000)
	Chicks	M and F	Corn oil	1,190 (847-1,670)
	Rabbits	F	Undiluted	819 (610-1,070)

ues given in the literature are included in table 3.

Toxic symptoms generally observed in animals made ill with this type of compound include loss of appetite, loss of weight, depression, roughness of coat, general tenseness, and muscular weakness particularly of the posterior quarters. Postmortem findings usually include irritation of the stomach of small animals and of the abomasum of ruminants, minor evidence of liver and kidney injury, and in some instances congestion of the lungs.

ORAL ADMINISTRATION OF REPEATED DOSES OF 2,4-D TO RATS AND CHICKS

Experiment 1, Rats. — Procedure. — Matched groups of 5 or 6 young adult female rats from the stock colony of this laboratory were fed 2,4-D five times a week for four weeks by intubation. The dosages employed were 0.0 (controls), 3.0, 10.0, 30.0, 100.0, and 300.0 mg./kg. administered as aliquots of olive oil solutions emulsified in about 2 ml. of 5 to 10 per cent aqueous gum arabic solution. The controls received appropriate doses of olive oil.

Results.—The control animals and those animals receiving 3.0, 10.0, and 30.0 mg./kg. of 2,4-D showed no adverse effects as judged by gross appearance and behavior, mortality, growth, hematological values, blood urea-nitrogen concentrations, organ weights, and gross and microscopic examination of the tissues.

Rats receiving 100.0 mg./kg. showed varying degrees of gastrointestinal irritation, slight cloudy swelling in the liver, and a depressed growth rate. Those receiving 300.0 mg./kg. failed rapidly and died. Severe gastrointestinal irritation was the principal adverse effect observed.

Experiment 2, Rats. — Procedure. — Matched groups of 5 young adult female rats from the stock colonies of this laboratory were placed on diets containing 0 (control), 100, 300, 1,000, 3,000, and 10,000 p.p.m. (parts per million by weight) of 2,4-D.

Results.—The controls and those animals receiving the diets containing 100 and 300 p.p.m. of 2,4-D for 113 days showed no adverse effects as judged by food consumption, growth, general appearance, mortality, blood urea-nitrogen concentrations, hematological examinations, organ weights, and gross and microscopic examination of the tissues.

Those on the diet containing 1,000 p.p.m. for 113 days suffered slight adverse effects characterized by depressed growth rate, excessive mortality, slightly increased liver weights, and slight cloudy swelling of the liver.

Those on the two higher diets were destroyed after twelve days as they were not eating and were rapidly losing weight. Examination revealed increased liver and kidney weights and slight pathological changes in these organs.

Experiment 3, Chicks.—Procedure.—Twelve New Hampshire Red chicks weighing from 210 to 340 Gm. each were divided into four groups of 3 chicks each and maintained for seven days on diets containing 0 (controls), 500, 1,000, and 3,000 p.p.m. of 2,4-D.

Results.—The only adverse effects apparent grossly were a reduction in food intake and a retarded growth rate in the birds fed the diet containing 3,000 p.p.m. of 2,4-D. No histopathological examinations were made.

ORAL ADMINISTRATION TO STEERS

Purpose.—Since essentially all of the toxicological information on 2,4-D and 2,4,5-T type herbicides was obtained using small animals and since the practical application of these materials involves exposure also to large animals, experiments were undertaken to ascertain if extrapolation of the data obtained on the laboratory animals to large animals was justified.

Esteron Brush Killer.—Since esteron brush killer (formulation of esters of 2,4-D and 2,4,5-T) is perhaps the widest used of the products discussed herein, it was chosen for study.

In the experiments outlined in the following paragraphs, administrations were all by intubation. Treated animals were constantly under observation and the ordinary clinical observations were routinely made. In general, only positive observations are reported.

Experiment 1.—A steer weighing 291 kg. was given a single dose of 1,000 mg./kg. No perceptible symptoms of toxicity were observed.

Experiment 2.—A steer weighing 295 kg. was given 1,000 mg./kg. of esteron brush killer on each of three successive days. General depression, decreased food and water intake, and decreased rumen motility were observed following the third dose. These symptoms became increasingly severe until the steer died on the third day following the last dose. Death was undramatic; no convulsions, struggling, or signs of pain were observed at any time. Necropsy revealed the following: Rumen contents were dry and smelled strongly of the

esteron; the abomasum was impacted and the contents of the intestines were entirely fluid; the mesenteric vessels were congested and the spleen was dark and shrunken.

Experiment 3.—A steer weighing 295 kg. was given 500 mg./kg. on each of two consecutive days. On the third day, the animal was off feed and rumen motility had essentially ceased. On the fourth day, the steer appeared perfectly normal and there were no discernible after-effects.

Experiment 4.—Another steer weighing 336 kg. was given 500 mg./kg. on each of three successive days. No toxic symptoms were observerd and the animal remained on full feed.

Experiment 5.—A steer weighing 250 kg. was given 100 mg./kg. of esteron brush killer on each of fifteen consecutive days without any outward appearance of adverse effects. The animal was killed for study forty-eight hours following the last dose. Gross examination of the internal organs revealed only slight petechial hemorrhage in the duodenum and a mild diffuse irritation in the abomasum. Histological examination of tissues from the lung, heart, spleen, adrenals, pancreas, thyroid, thymus, bladder, and lymph nodes revealed no abnormalities. Sections from the liver revealed small areas of focal hemorrhagic necrosis surrounded by areas of fatty degeneration. In the kidneys, very slight interstitial edema and congestion in the medulla and cortical medullary region were observed. Although gross examination of the gastrointestinal tract revealed mild irritation in the duodenum and abomasum, sections of these organs failed to indicate any changes of significance.

Kuron (H-1078).—Because of the current interest in derivatives of chlorinated phenoxypropionic acid, one of the more promising formulations, kuron (table 1), was included in the work with cattle. A steer weighing 250 kg. was given 100 mg./kg. of kuron on each of fifteen consecutive days. No adverse effects were noted when judged by general appearance, behavior, and weight gain. The animal was killed for examination forty-eight hours following the last dose. Gross examination of the in-

TABLE 4—Summary of Acute Oral Toxicity of Various Herbicidal Formulations (Dow)

Material	Species	Sex	Vehicle	l.d.$_{50}$ (19/20 confidence limits) (mg./kg.)
2,4-Dow weed killer	Guinea pigs	F	Water	Range 2,000 (1,000-3,000)
2,4-Dow weed killer (formula 40)	Rats	F	Water	Range 850 (700-1,000)
Esteron 44	Rats	M	Olive oil	Range 650 (300-1,000)
Esteron 245 (old)	Rats	F	Emulsion in water	Range 1,000 (300-2,000)
Esteron 245 (new)	Rats	M	Olive oil	Range 800 (600-1,000)
Esteron ten-ten	Rats	F	Olive oil	760 (660-860)
Brush killer 50-50	Rats	F	Emulsion in water	1,070 (700-1,650)
	Guinea pigs	F	Emulsion in water	1,160 (820-1,630)
	Mice	F	Corn oil	800 (624-1,070)
	Rabbits	F	Undiluted	Range 1,420 (500-2,000)
	Chicks	M and F	Undiluted	4,000 (2,700-5,900)
Brush killer T	Rat	F	Emulsion in water	1,200 (783-1,850)
	Guinea pigs	F	Emulsion in water	1,410 (875-2,290)
	Mice	F	Olive oil	1,230 (938-1,620)
	Rabbits	M	Undiluted	849 (604-1,190)
	Chicks	M and F	Undiluted	2,000 (1,350-2,960)
Brush killer 76	Rats	F	Corn oil	Range 750 (500-1,000)
Brush killer 76E	Rats	F	Corn oil	Range 500 (250-1,000)
Esteron brush killer (old)	Rats	M	Emulsion in water	Range 1,000 (300-3,000)
Esteron brush killer (new)	Rats	M and F	Corn oil	860 (800-930)
	Guinea pigs	M	Corn oil	1,220 (1,040-1,430)
	Guinea pigs	F	Corn oil	1,600 (1,390-1,840)
	Rabbits	M and F	Corn oil	960 (790-1,160)
	Chicks	M and F	Corn oil	Range 2,000 (1,000-3,000)
	Steers		Undiluted	Greater than 1,000

ternal organs revealed no abnormalities. Histological examination of tissues from the lung, heart, liver, spleen, pancreas, thymus, bladder, lymph nodes, rumen, reticulum, omasum, and abomasum revealed no abnormalities. Very slight interstitial edema and congestion was observed in the medulla and cortical medullary regions of the kidney, but since this has frequently been observed in untreated animals, its significance is questionable.

DISCUSSION

Significance of Experimental Results.—Study of the data presented in table 3 indicates that the various forms of 2,4-D and 2,4,5-T all fall in the same range of toxicity for rats, mice, guinea pigs, and rabbits. The dog appears to be somewhat more susceptible to these materials than the other species studied, and chicks appear to be more tolerant. It is also apparent that MCP (2-methyl-4-chlorophenoxyacetic acid), silvex (2-[2,4,5-trichlorophenoxy] propionic acid), and their derivatives are less toxic acutely than the corresponding derivatives of 2,4-D and 2,4,5-T. The $l.d._{50}$ values for 2,4-D and 2,4,5-T and their common derivatives are in the range of 300 to 1,000 mg./kg. for the rat, mouse, guinea pig, and rabbit, whereas for MCP and silvex, the corresponding values range from 600 to 1,400 mg./kg.

The data presented in table 4 show that the acute oral toxicity of the commercial formulations, in general, tends to be proportional to that which might be expected from their content of active ingredients. Thus, it appears that the "inert ingredients" present in these formulations are less toxic than the active agents. Furthermore, the "inerts" do not appear to exert a potentiating effect upon the toxicity of the active materials. For the commercial formulations studied, the $l.d._{50}$ values for rats, mice, guinea pigs, rabbits, and cattle range from 500 to 2,000 mg./kg.; for chicks the range is from 2,000 to 4,000 mg./kg.

The results of repeated oral administrations indicate that 2,4-D and 2,4,5-T can be tolerated without adverse effects in doses only slightly smaller than those which cause toxic effects when given only once. This fact demonstrates that these materials have a low degree of chronicity.

The studies with cattle have demonstrated the similarity in susceptibility of cattle and small laboratory animals to this type of herbicidal materials. It has also been demonstrated that cattle are distinctly more tolerant of this type material than dogs.

The following conclusions with regard to cattle seem justified:

1) A daily dose of 30 mg./kg. probably can be tolerated for prolonged periods without adverse effect.

2) A daily dose of 100 mg./kg. would not be expected to cause any ill effects unless exposures were continued for a week or longer. However, if exposures were continued, some liver and kidney injury, and perhaps some gastrointestinal irritation could occur. Death or serious illness would not be anticipated.

3) A daily dose of 500 mg./kg., if continued for several days, could result in serious effects. A single dose of 500 mg./kg. would not be likely to cause serious effects.

4) A single dose of 1,000 mg./kg. may or may not cause illness. Repeated doses of this size are very likely to cause death.

Evaluation of Direct Hazard to Livestock.—In actual practice, esteron brush killer (a formulation of esters of 2,4-D and 2,4,5-T) and similar formulations are not ordinarily used on areas containing appreciable forage at a rate greater than 2 quarts per acre or 47 mg./sq. ft.

Assuming that *all* of the herbicide applied at this dosage rate is deposited on edible forage, an animal weighing 350 kg. (770 lb.) would have to consume *all* the forage on 744 sq. ft. of treated area to acquire a dose of 100 mg./kg. of the herbicidal formulation. Such a dose could be tolerated daily for a number of days without any serious effects. Based on the same assumption, it would be necessary for the animal to graze completely an area ten times as large, or 7,440 sq. ft. (0.17 acres) in order to acquire an acutely toxic dose (approximately 1,000 mg./kg.).

Since these assumptions are based upon *complete* availability to livestock of *all* the herbicide applied, an obviously impossible condition, it is apparent that there is little, if any, direct hazard to livestock or wildlife foraging areas treated with herbicides of the type described herein. This has been confirmed by the extensive use of these ma-

terials over a number of years without any proved cases of adverse effects.

Evaluation of Indirect Hazard to Livestock.—There has been much discussion of the possibility that spraying with these herbicides might cause some plants to become toxic, toxic plants to become more toxic, and ordinarily nonpalatable plants to become palatable. Considerable investigation of these possibilities has resulted in the general conclusion that from a practical standpoint these suppositions were more fanciful than factual.

In 1950, Willard [11] of Ohio State University and Agricultural Experiment Station pointed out that the spraying of certain types of plants with 2,4-D increased their nitrate content to hazardous levels.

The leaves of sugar beets accidentally sprayed with 2,4-D were reported to have caused the poisoning of some livestock. However, since 2,4-D is never knowingly used on sugar beets, this does not appear to present a serious practical hazard. Other weeds such as lambsquarter, pigweed, and smartweed have also been reported as containing increased quantities of nitrate after treatment with 2,4-D. Since livestock do not ordinarily eat these species, it would seem that the hazard presented by the accumulation of nitrate in these plants is slight. The lack of reported cases of nitrate poisoning would seem to substantiate this supposition.

Wild cherry is recognized as a hazardous plant to have in grazing areas. Couch [8] has suggested that the wilting of wild cherry leaves results in the hydrolysis of cyanogenic glucosides to form free hydrocyanic acid (HCN) and, therefore, that cut wild cherry is much more hazardous to livestock than the growing plants. By analogy, it has been postulated that wild cherry leaves wilted as a result of treatment with 2,4-D and 2,4,5-T herbicides likewise might be more toxic than growing leaves. Barrons and Lynn [9] and Grigsby and Ball,[10] working independently, demonstrated clearly that the HCN content of wild cherry leaves sprayed with esteron brush killer was actually lower than the content of unsprayed leaves.

Dr. Willard also has called to attention the possibility that treatment with 2,4-D type herbicides might make certain naturally poisonous plants more palatable or even attractive to livestock. This is a possibility but, so far, it has not been established as a fact.

In evaluating the hazard associated with the forementioned possibilities, Dr. Willard in 1951 [12] stated as follows: "In the first place, literally dozens of carefully controlled feeding experiments, both with 2,4-D and 2,4,5-T directly and with vegetation sprayed with them, have uniformly and without exception failed to show any poisonous effects on any kind of livestock.

"In the second place, literally millions of acres of pastures with livestock on them have been sprayed in the last six or seven years without injury to livestock. I am sure there is no experimenter here who has not used 2,4-D in occupied pastures. In our state, one company over a period of four years has sprayed 60,000 miles of roadside in twenty-six counties. During that time, he has had only five reports of suspected poisonings, none of them substantiated, 2 of swine, 2 of cattle, 1 of sheep. A company in Pennsylvania, which has sprayed 10,000 acres of right-of-ways per year, reports just 4 cases, only 1 of which was not completely cleared up as due to other causes, in five years of work."

In 1953, Dr. S. N. Fertig [13] of Cornell University summarized the situation regarding poisoning as follows, "There are no known cases of actual herbicidal poisoning from field application of presently used herbicides marketed as nonpoisonous.* There is no sound toxicological evidence available of the presently publicized nitrate-phenol and nitrite poisoning as a common cause of death. True, nitrate and nitrite poisoning has occurred in cattle, sheep, and swine from eating plants grown on high nitrate soils or from other feed sources. None of these, however, have been definitely traced or shown to be caused by herbicidal treatment. All the alleged cases of herbicidal poisoning of livestock and wildlife that have been definitely diagnosed have been caused by one of the following: (1) lead, (2) arsenic, (3) hardware disease, (4) poisonous plants, (5) old age, (6) parasites, (7) drowning, (8) poor marksmanship, (9) contaminated food, and (10) injection or oral dosage of some medicine or drug. In all cases that have been carefully surveyed, even though the herbi-

* Dr. Fertig's classification of herbicides marketed as non-poisonous includes such materials as those discussed in this paper.

cide has been associated with the trouble, it has in no case been directly or indirectly related to the deaths reported."

SUMMARY AND CONCLUSIONS

1) The acute oral $l.d._{50}$ values for 2,4-D, 2,4,5-T, and their various derivatives commonly used in herbicidal preparations fall in the range of 300 to 1,000 mg./kg. for rats, mice, guinea pigs, and rabbits. Generally, dogs are more susceptible and chicks are more tolerant of this type of material. On a weight basis, the toxicity to cattle appears to be quite similar to the toxicity to ordinary laboratory animals.

2) The acute oral $l.d._{50}$ values for commercial formulations of 2,4-D and 2,4,5-T type materials are approximately proportional to their content of active ingredient. The "inert ingredients" do not appear to contribute materially to the oral toxicity of the formulations.

3) Data available indicate that the 2,4-D and 2,4,5-T type herbicides h ve a low chronicity.

4) MCP (2-methyl-4-chlorophenoxyacetic acid) and silvex (2-[2,4,5-trichlorophenoxy] propionic acid) and their herbicidal derivatives appear to be slightly less toxic than the corresponding 2,4-D and 2,4,5-T type materials.

5) The hazard to livestock and wildlife associated with the use as recommended of herbicides containing 2,4-D, 2,4,5-T, MCP, and silvex is negligible. It should be recognized, however, that toxic amounts of these materials can be obtained if animals have access to spray tanks or other containers of the materials.

References

[1] Bucher, Nancy L. R.: Effects of 2,4-Dichlorophenoxyacetic Acid on Experimental Animals. Proc. Soc. Exptl. Biol. and Med., *63*, (1946): 204-205.

[2] Hill, Edwin C., and Carlisle, Harold: Toxicity of 2,4-Dichlorophenoxyacetic Acid for Experimental Animals. J. Indust. Hyg. and Toxicol., *29*, (1947): 85-95.

[3] Bjorn, Melvin K., and Northen, Henry T.: Effects of 2,4-Dichlorophenoxyacetic Acid on Chicks. Science, *108*, (1948): 479-480.

[4] Drill, Victor A., and Hiratzka, Tomiharu: Toxicity of 2,4-Dichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyacetic Acid. A Report on Their Acute and Chronic Toxicity in Dogs. Arch. Indust. Hyg. and Occup. Med., *7*, (1953): 61-67.

[5] Mitchell, J. W., Hodgson, R. E., and Gaetjens, C. E.: Tolerance of Farm Animals to Feed Containing 2,4-Dichlorophenoxyacetic Acid. J. Anim. Sci., *5*, (1946): 226-232.

[6] Grigsby, B. H., and Farwell, E. D.: Some Effects of Herbicides on Pasture and on Grazing Livestock. Michigan Agric. Exper. Station Quart. Bull., *32*, (1950): 378-385.

[7] Litchfield, J. T., Jr., and Wilcoxon, F.: A Simplified Method of Evaluating Dose-Effect Experiments. J. Pharmacol. and Exper. Therap., *96*, (1949): 99-113.

[8] Couch, James F.: Poisoning of Livestock by Plants that Produce Hydrocyanic Acid. U.S.D.A. Leaflet 88, 1940.

[9] Lynn, G. E., and Barrons, K. C.: The Hydrocyanic Acid (HCN) Content of Wild Cherry Leaves Sprayed With a Brush Killer Containing Low Volatile Esters of 2,4-D and 2,4,5-T. Proc. Sixth Ann. Meet. Northeast. Weed Control Conf., (1952): 331-333.

[10] Grigsby, B. H., and Ball, C. D.: Some Effects of Herbicidal Sprays on the Hydrocyanic Acid Content of Leaves of Wild Black Cherry (*Prunus Serotina Ehrhart*). Proc. Sixth Ann. Meet. Northeast. Weed Control Conf., (1952): 327-330.

[11] Willard, C. J.: Indirect Effects of Herbicides. Proc. Seventh Ann. Meet. North Central Weed Control Conf., (1950): 110-112.

[12] Willard, C. J.: The Status of Herbicidal Poisoning. Proc. Eighth Ann. Meet. North Central Weed Control Conf., (1951): 86-89.

[13] Fertig, S. N.: Herbicidal Poisoning of Livestock. Suppl. Proc. Seventh Ann. Meet. Northeast. Weed Control Conf., (1953): 44-47.

The Illustration of the Morphology of *Vibrio Fetus* by Electron Microscopy

HARRY E. RHOADES, M.S.

Urbana, Illinois

LITERATURE CITED

The characteristics of *Vibrio fetus* were first described in England by a committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic abortion in sheep [5] and in an appendix to this report by McFadyean and Stockman.[3] Smith and Taylor [7] isolated and described an organism from the fetal membranes of cattle in 1919 which they named *Vibrio fetus*. Plastridge *et al.*[4] and Ryff and Lee [6] have also described the organism.

MATERIAL AND METHODS

One ovine and one bovine strain of *Vibrio fetus* were used in this study. Cultures were transferred to fresh tryptose agar slants at three-day intervals and incubated at 37 C. under 10 per cent carbon dioxide.

In the preparation of *Vibrio fetus* cultures for electron microscopy, a small amount of the culture was removed from an agar slant and suspended in 0.05 M. phosphate buffer (pH 7.0). A small drop of the suspension was placed on a collodion coated, stainless steel grid of 200 mesh and exposed to the vapor of a 2 per cent solution of osmium tetroxide for twenty minutes. After fixation, the preparation was dried and then washed with distilled water to remove the salt crystals. The washed specimen was redried and shadow cast with either a gold-chromium alloy or palladium. The angle of shadow casting was about 18 degrees. The pictures were taken at magnifications of 3,000 to 10,000.

The cultures on initial isolation were made up almost entirely of comma and S-shaped rods (fig. 1 and 2). These curved rods measured about 0.5 μ wide and 1.5 to 5.0 μ long. In young cells, the cytoplasm of the cell was homogenous (fig. 1, 2, and 3). The organism was motile by means of a single, polar flagellum or by bipolar flagella. The latter arrangement was found predominantly on the S-shaped cells and on some filamentous forms (fig. 4 and 7).

On succeeding transfers, the organism became more filamentous and the cytoplasm had a granular appearance. The granules appeared in the form of dots at one end of some of the comma-like cells (fig. 5). In some long forms they were arranged at fairly regular intervals (fig. 6). According to Davis and associates,[1] refractile granules found in bacteria may be mitochondria or nuclei.

No segmentation of the filamentous forms was discernible in this work (fig. 7 and 7a).

When the bovine strain of *Vibrio fetus* was transferred on agar slants that were allowed to dry somewhat before inoculation and transfers were made at four-day intervals, a coccoid form appeared. This coccoid form occurred in old cultures and under unfavorable conditions for growth. It had a single flagellum and the cytoplasm was granular (fig. 8). A saline suspension of a culture containing many coccoid forms auto-agglutinated. The first report of this coccoid form was by McFadyean and Stockman.[3] Firehammer and Marsh [2] mentioned the undesirability of using a culture containing this form for antigen production.

Presented at the Fifty-third General Meeting of the Society of American Bacteriologists, San Francisco, Aug. 10-14, 1953.

From the Department of Veterinary Pathology and Hygiene, University of Illinois, Urbana. The author wishes to acknowledge the assistance of Mr. A. E. Vatter in the preparation of the electron photomicrographs.

Legends for Illustrations on Opposite Page

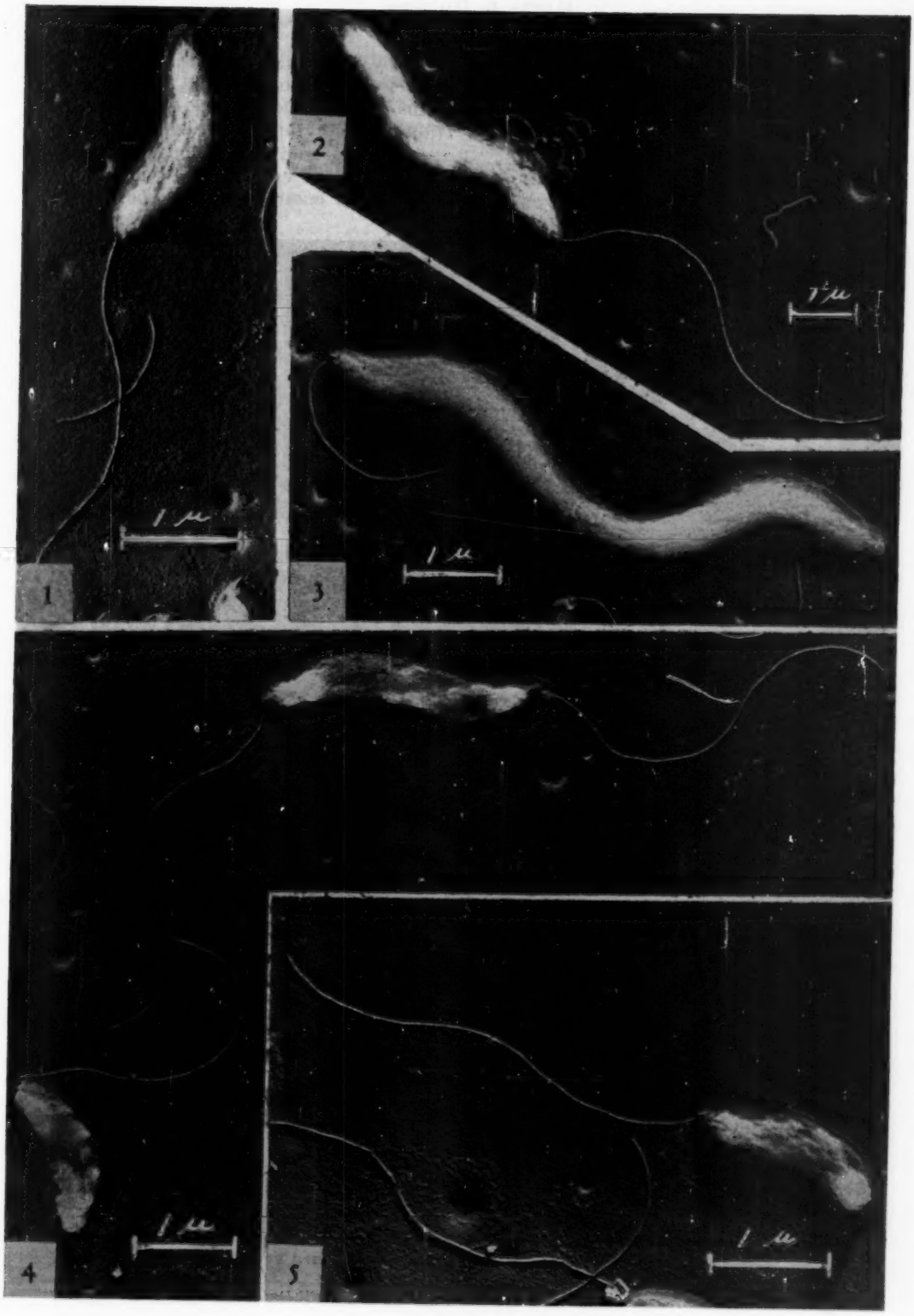
Fig. 1—Comma-shaped Vibrio fetus cell measuring 0.5 μ wide and 2.0 μ long.

Fig. 2—S-shaped V. fetus cell measuring 0.8 μ wide and about 5.0 μ long.

Fig. 3—S-shaped V. fetus cell with homogenous cytoplasm surrounded by the cell wall.

Fig. 4—In the lower left corner is a comma-shaped V. fetus cell with a single polar flagellum. At the top of the picture is an S-form with bipolar flagella.

Fig. 5—Comma-shaped V. fetus cell with a granule in the form of a dot at the right end of the cell.


1
1 μ
2
1 μ
3
1 μ
4
1 μ
5
1 μ

The bovine strain used in this study produced the various forms more readily than the ovine strain.

SUMMARY

The variations in the morphology of *Vibrio fetus* are illustrated by electron microscopy. On initial isolation, the organisms were predominantly comma and S forms with homogeneous cytoplasm. On transfer the cytoplasm became granular in appearance, and very long filamentous forms were observed. The comma forms had a single polar flagellum, whereas many

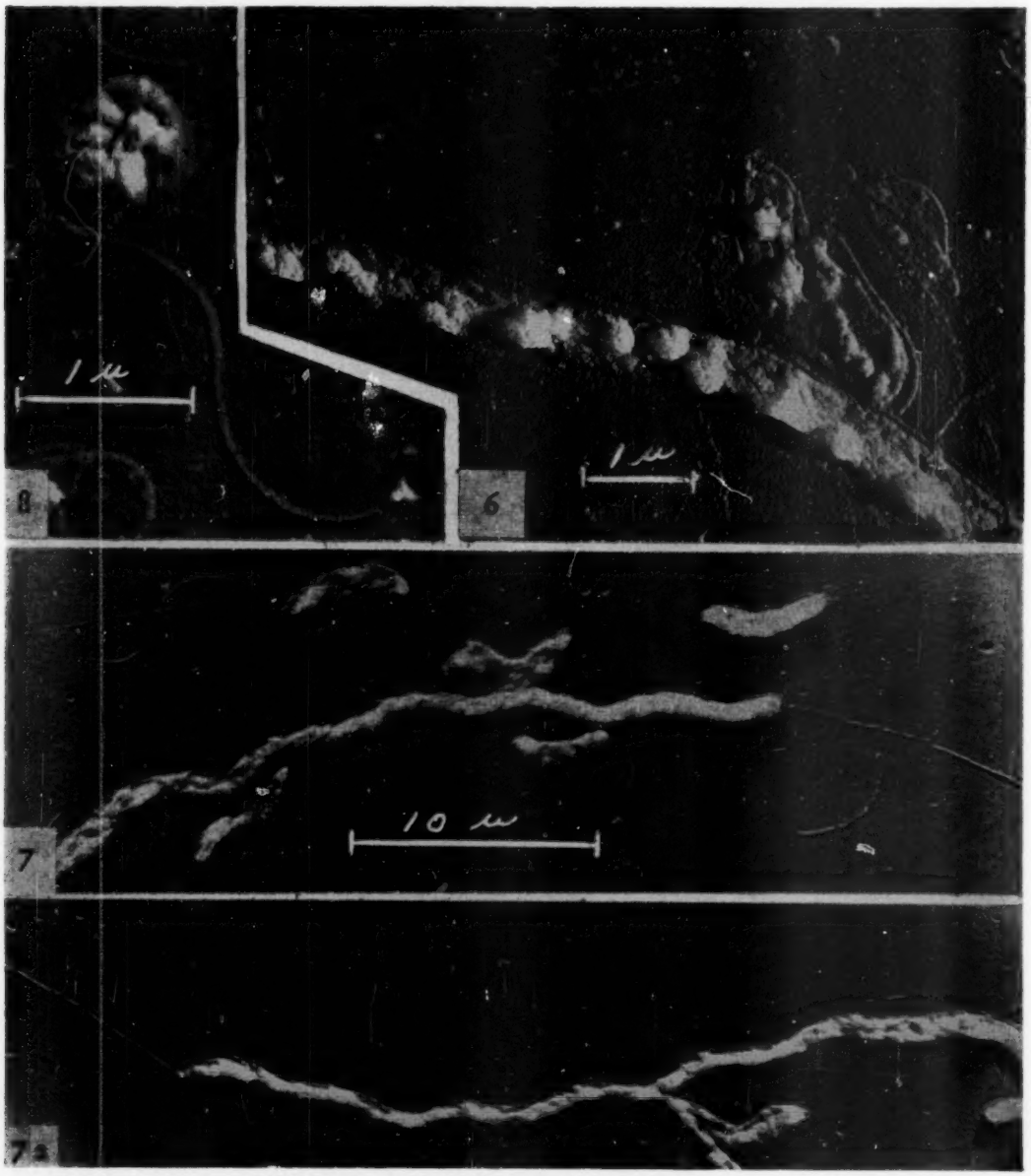


Fig. 6—Long form of Vibrio fetus cell with granules arranged at regular intervals along the filament.

Fig. 7 and 7a—Nonsegmented long filamentous form of Vibrio fetus cell with bipolar flagella.

Fig. 8—Coccoid form of Vibrio fetus cell with granular cytoplasm and a single flagellum.

of the S forms had bipolar flagella. Prolonged incubation and transfer to dry slants produced a coccoid form with a single flagellum. This form auto-agglutinated.

References

[1] Davis, J. C., Winterscheid, L. C., Hartman, P. E., and Mudd, Stuart: Cytological Investigation of the Mitochondria of Three Strains of *Salmonella typhosa*. J. Histochem. and Cytochem., *1*, (1953): 123-137.

[2] Firehammer, B. D., and Marsh, H.: Preparation of Antigen for Detection of Agglutinins for *Vibrio Fetus*. Am. J. Vet. Res., *14*, (1953): 392-395.

[3] McFadyean, J., and Stockman, Stewart: Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizootic Abortion. Appendix to Part III. Abortion in Sheep. 1913. House of Common Sessional Papers, *12*, 1914.

[4] Plastridge, W. N., Williams, L. F., Easterbrooks, H. L., Walker, E. C., and Beccia, R. W.: Vibriosis in Cattle. Storrs Agric. Exper. Sta. Bulletin 281. Dec., 1951.

[5] Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizootic Abortion. Part III. Abortion in Sheep. 1913. House of Commons Sessional Papers, *12*, 1914.

[6] Ryff, J. F., and Lee, A. M.: *Vibrio Fetus* in Sheep. Am. J. Vet. Res., *6*, (1945): 149-158.

[7] Smith, Theobald, and Taylor, M. S.: Some Morphological and Biological Characters of the Spirilla (*Vibrio Fetus*, n. sp.) Associated with Disease of the Fetal Membranes in Cattle. J. Exper. Med., *30*, (1919): 229-311.

Marginal Punched Cards in Veterinary Research

C. W. SCHWABE, M.S., D.V.M., and L. R. DAVIS, Ph.D.

Auburn, Alabama

THE MARGINAL punched card is useful to the researcher in veterinary medicine for filing reprints, compilation of bibliographies, filing projection and microscope slides, maintenance of autopsy and laboratory records, organization of lecture notes, taxonomic keying of organisms, and in the preparation of papers and books for publication. The imaginative user might add to this list indefinitely; for once acquainted with the basic punched card techniques, additional applications suited to his particular interests and routine will become increasingly evident. The busy practitioner might adopt the method as a simple system of filing case records.

Marginal punched cards are not new but their widest usage has previously been in the fields of chemistry and library science. Only in recent years have many biologists become even aware of their existence, although the various chemical research journals contain numerous papers discussing their usefulness in that field.

The basic principle of marginal punched cards is similar to that of the more familiar IBM*-type card, that is, various categories of useful information are punch-coded upon each card, and all cards pertaining to any particular category of information may be quickly and easily secured from the file by a mechanical sorting process. Unlike the IBM system, however, marginal punched cards are especially suited to individual and small laboratory usage because mechanical hand-sorting rather than machine sorting is employed, and because the necessary equipment is neither complicated nor expensive. One needs a hand punch, tumbler (sorting needle), marginal punched cards, and a booklet of gummed, perforated hole-repair stickers.**

The basic techniques may be demonstrated most readily by illustrating an actual usage. Many veterinary researchers maintain a collection of color, or other type, projection slides pertaining to their work. These may be useful to illustrate classroom lectures, papers presented at scientific meetings, talks before lay groups, and a variety of other purposes. To secure maximum usefulness from a slide collection, a pathologist may wish to have it filed under such categories as specific disease, animal species, organ involved, type of pathology, etiological agent, and others. Using conventional systems of filing, he may do either of two things: (1) break down the slide filing drawers or boxes into the categories desired and file a duplicate of the slide under each category; for instance, a slide portraying one lesion might be filed under sterility, bovine, testis, fatty change, Brucella spp., et cetera, requiring at least five copies of the slide, or (2) the slides might be filed numerically and a conventional card file broken down into each category as listed above. This would require typing five or more cards for each slide. Although both of these methods would permit maximum use of the slide collection, each has obvious disadvantages involving expense, space, or labor, all of which are important factors for the consideration of the busy researcher.

Marginal punched cards might be used to handle the situation in this way. File slides numerically as they come to hand. Prepare a code card (fig. 1) for the categories of information desired from the collection, and devote one card to each slide in the collection, punching the holes corresponding to the code. Write the number of each slide upon its particular card. The card file then is entirely random without any attempted semblance of sequence. Figure 1 shows a card punched for "sterility, bovine, testis, fatty change, Brucella spp." (Temporarily ignore those holes pertaining

Contribution No. 414 approved by Committee on Publications, School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn.

From the Dept. of Bacteriology (Schwabe), School of Veterinary Medicine, Alabama Polytechnic Institute, Auburn, and the Regional Animal Disease Research Laboratory (Davis), Agricultural Research Service, U. S. Department of Agriculture, Auburn, Ala.

Dr. Schwabe's present address: Harvard School of Public Health, Boston, Mass.

* International Business Machines.

** The accessories and the marginal punched cards (standard form Y9) were purchased from Charles R. Hadley Co., 330 N. Los Angeles St., Los Angeles, Calif.

to specific disease and etiological agent.) Now should one desire all slides illustrating lesions of the bovine testis, for instance, pass the tumbler through the hole coded "testis," and this would drop all of the cards pertaining to the testis. The needling technique is shown in figure 2. Then by passing the tumbler through the hole coded "bovine" in the dropped cards, all cards pertaining to the bovine testis will be sorted out. All the slides on the bovine testis may be secured from the file by referring to the slide number on each dropped card. The sorting process could have been continued further by successively needling "fatty change," "Brucella spp." et cetera. Needless to say, the explanation of these techniques consumes much more time than their actual operation.

What has just been described is called "sorting by direct coding," that is, assigning a numbered hole in a code to each category desired, such as was done under animal species, organ involved, and type of pathology. This type of simple coding is limited only by the number of holes available on the card (for instance, 91 numbered holes on the 5- by 8-in. card and 51 holes on a 3- by 5-in. card). In such categories as specific disease and etiological agent, however, one can see that the number of working subdivisions desired may exceed the number of holes available. In situations such as this, one must resort to one of several systems of indirect coding. Notice in figure 1 that, in addition to a continuous numbering of the holes around the margin, the holes are divided into series of four, each series numbered 7, 4, 2, 1. These series are space savers; with them one may punch any number from 0 to 9 in just four spaces (none, 1, 2, 2 plus 1, 4, 4 plus 1, 4 plus 2, etc.). In punching higher numbers, 10-99, employ a second series of four to the left; for 100-999, a third series, and so on *ad infinitum*, each series reading from the right, "units, tens, hundreds, thousands," et cetera. Here the potentialities of marginal punched cards become obvious; with only 12 holes, 999 categories of information can be coded for easy sorting. In the above example, specific disease and etiological agent were coded by this method. A number on the master code is assigned to each disease and each etiological agent desired; *i.e.* 1 for "anthrax" . . . to 80 for "sterility," etc., on the lower edge, and 1 for "Anaplasma spp." . . . to 467 for "Brucella spp." on the upper edge (fig. 1).

The sorting technique for indirect coding is the same as for direct coding, except the cards usually must be needled several times or with several tumblers simultaneously. When properly needled the desired number will drop. It takes but slight mathematical curiosity to see that with certain numbers in indirect coding, in addition to the desired cards, some unwanted numbers may also fall (this is largely a result of the 7, 4, 2, 1 combination). Practically speaking, this seeming disadvantage of indirect coding is seldom an inconvenience, but it should encourage direct coding wherever applicable. This example of the use of marginal punched cards for a color slide file illustrates most of the basic techniques. Of course, dates and many other types of useful information could also have been coded. There are several modifications of direct coding which extend its usefulness, and one will be considered presently.

Marginal punched cards are available in a large variety of sizes, some of which are standard (3- by 5-in., 5- by 8-in., etc.), and others which must be made to order. The size of the card selected for any particular use will depend upon the amount of direct coding to be done or the amount of written material to be included on the card. For the color slide file where the slide number was the only thing written, a smaller card could have been used. In compiling bibliographies or lecture notes, a larger card, such as in figure 1, would be more useful. Cards are available in varied rag content which determines their durability. Good grade cards cost as little as 1 cent each in lots of 500. More complicated varieties include two or more rows of punched holes. Some manufacturers will color code the margins, or the card may be printed on colored stock.

An extended modification of direct coding will be illustrated with a reprint file. Figure 3 shows a suitable code card. The top edge on the right is directly coded for type of etiological agent, and on the left for animal species. The bottom edge of the card (temporarily ignore the sides) is coded for specific diseases by a modified

direct code. That is, one must needle a category on the top right *before* selecting the specific disease on the bottom, because the bottom is used for *each* category

TABLE 1—Combination of the Letters "O-I-E-C-B" as Used to Make an Alphabetical Indirect Code for Authors of Reprints

A—None	G—EC	Ma —IE	Ou —OC	U —OI	
B—B	H—ECB	Mc —IEB	R —OCB	V —OIB	
C—C	I —I	Mac—IEB	Sa —OE	W—OIC	
D—CB	J —IB	M —IEC	Sch—OEB	X —OICB	
E—E	K—IC	N —IECB	S —OEC	Y —OIE	
F—EB	L—ICB	O —O	T —OECB	Z —OIEB	
		P —OB			

of etiological agent coded. For instance, if "leptospirosis" is wanted, first needle "bacteria," and *then* needle 60 in the dropped cards, which is leptospirosis *under* bacteria. If 60 were needled first, disease number 60 under each type of etiological agent would drop. The number of specific diseases that could be indexed in this way would be 30 (number of bottom holes) times the number of principle categories (top right). If this were not a sufficient number the modified direct coding could be extended around the right side, or else an indirect code could be utilized as was done for specific disease in the color slide file. This technique of modified direct coding greatly increases the usefulness of the card.

Now consider the sides of the card in figure 3. One may wish to code such things as author, date, and such divisions of disease as "diagnosis," "prophylaxis," "treatment," and "control," to name but a few. A single hole may be reserved for a special bibliography to be located *in toto*, such as all references to be included in a doctoral dissertation, or all the reprints pertaining to diseases in one locality. This latter service from a reprint collection requires but a single punch. For the paper's date use indirect coding, and for the author's name use an alphabetical code of which there are several. One that is commonly used is given in table 1, and illustrated in figure 3. Usually, only the

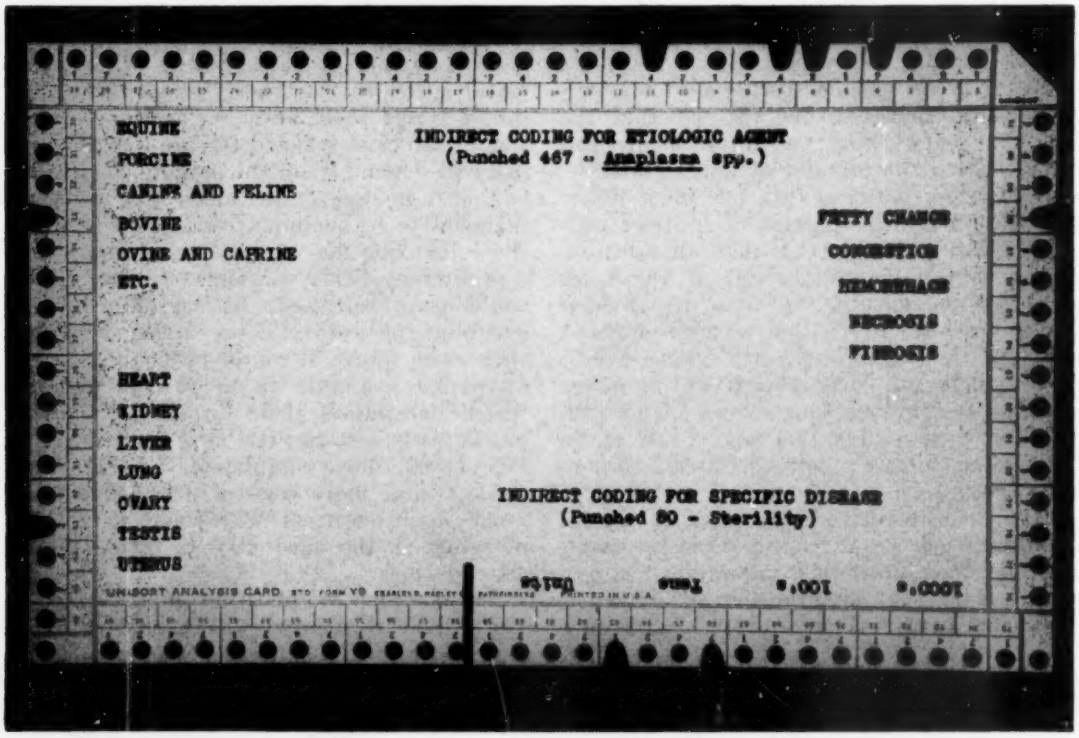


Fig. 1—Sample code card for a color slide file, directly coded for species, organ, type of pathology, and indirectly coded for etiological agent and specific disease. Standard marginal punched card, 5 by 8 inches.

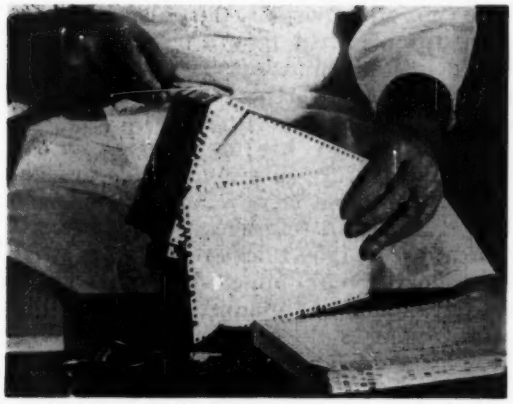


Fig. 2. Hand-sorting cards by using a tumbler. Desired punched cards drop as tumbler is raised. The other necessary equipment, margin punch, cards, and repair stickers, are shown.

first two or three letters of the author's name are coded. Figure 3 is correctly punched for the following paper:

> Alicata, J. E., and V. Breaks: Incidence of Leptospirosis Among Dogs in Honolulu as Determined by Serological Agglutination Tests. J. Washington Acad. Sci., *32*, (1942): 305-308.

In a typical reprint file, the bibliographical reference as above, plus the number assigned to the reprint, would be typed on the card. As the reprints are received they are filed by number without any regard to subject, author, et cetera. In the same card file, one might desire to include references to papers not in his library; in such cases, the marginal punched cards provide ample room on both sides for abstracting.

The form that is presently employed by a laboratory for such cases as autopsy and clinical laboratory reports and records could be printed or mimeographed directly on the cards. Marginal punched cards can end searching through piles of individual records for such things as all the blood urea nitrogen determinations for 1947-1953; just needle, and there they are.

The efficiency of the system is but slightly impaired by mounting clippings, photo-

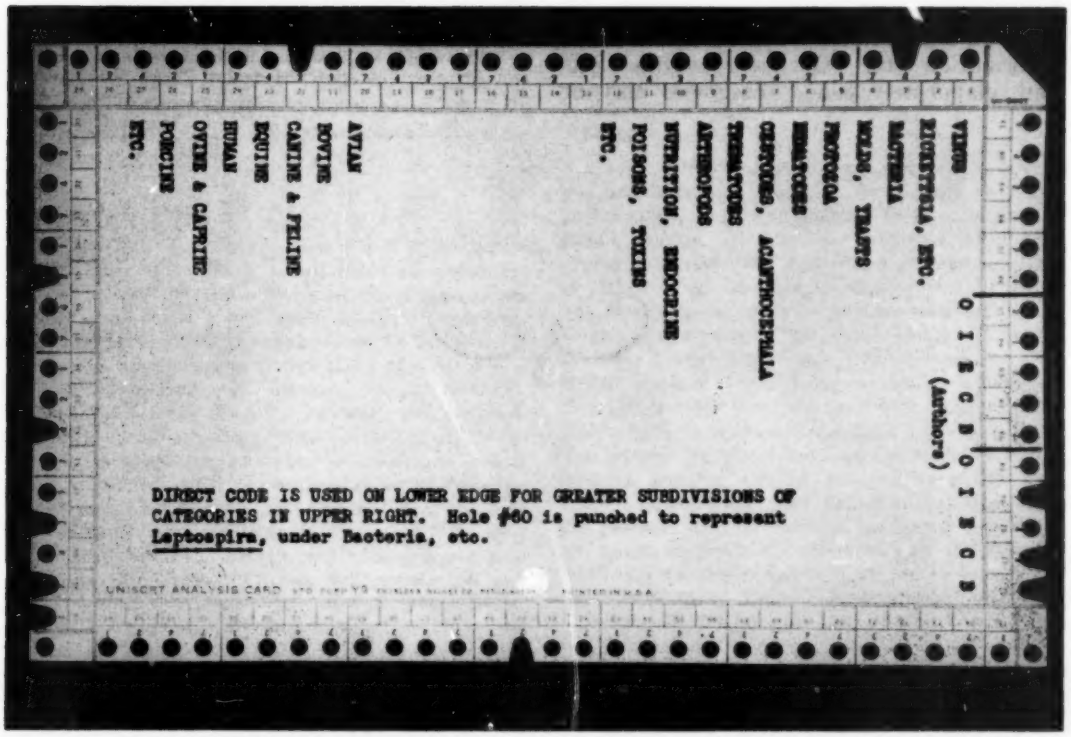


Fig. 3—Sample code card for reprint file, directly coded for species and type of etiological agent, indirectly coded for author's name and date of publication, and coded by a modified direct technique for specific disease.

graphs, or conventional file cards on the marginal punched cards (best done with photographic mounting tissue to reduce warping), and its usefulness for certain purposes might be increased thereby.

To illustrate one last usage, consider briefly the taxonomic key. Suppose it is desired to identify the ten species of coccidia which attack cattle in the southern states. Assign a direct code number to each characteristic used in identifying the species. For example, (1) colorless, (2) yellow, (3) brown, (4) yellow-green, et cetera, . . . (8) smooth wall, (9) some with mammillate walls, (10) distinctly thickened wall, . . . (15) no micropyle, (17) polar cap, (18) spherical, et cetera. A separate card should be used for each species, with numbers punched for each characteristic. Two or three sorting motions of the key cards are usually sufficient for identifying unknown species.

Before starting to work on a marginal punched-card system, one or two cautions are in order. First, keep it simple until sure of what information is needed; do not be in a hurry to make up an elaborate code and thus waste half the holes with information you will never needle. Second, elaborately coded categories may be such minute divisions that they will be more trouble than they are worth; there is a point of diminishing returns to watch for where hand-sorting of a few cards is easier than needle-sorting.

Punched card bibliographies of the world's literature in certain fields are becoming available on a subscription basis. For example, all references to electron microscopy for 1950-1952 are obtainable on approximately 700 punched cards and succeeding issues are planned.[1] Perhaps the same system could be utilized in fields of veterinary research.

The authors have covered only a few of the most useful techniques for veterinary research workers. There are simple systems for putting cards in alphabetical order, there are triangular and other codes, and many other published methods. The references [2,3] listed sum up many of these; they also include names and addresses of some of the manufacturers.

References

[1] Anonymous: News and Notes. Science, *118*, (1953): 378.

[2] Casey, R. S., and Perry, J. W.: Punched Cards. Reinhold Publishing Co., New York, N. Y., 1951.

[3] McGaw, H. F.: Marginal Punched Cards in College and Research Libraries. Scarecrow Press, Washington, D. C., 1952.

The Influence of Cobalt on the Action of Antibiotics

J. C. TRACE, D.V.M., and G. T. EDDS, D.V.M., Ph.D.

Fort Dodge, Iowa

MASTITIS caused by *Streptococcus agalactiae* has become less of a dairy herd problem since the advent of antibiotics, particularly penicillin. However, there has been a percentage increase in the incidence of mastitis caused by other streptococcal species, staphylococci, and coliform organisms.[1] Consequently, much work is being carried on to develop a formulation which would be more effective against the complete spectrum of bacteria associated with mastitis.

Streptomycin was found to increase the effectiveness of penicillin against mastitis pathogens and more recently sulfisoxazole and sulfathiazole were shown to potentiate the penicillin-streptomycin combination against some of the more resistant organisms associated with mastitis.[2,3] This present work was initiated with the objective of producing an antibiotic combination of even greater effectiveness. Since previous reports indicate that certain of the antibiotics are potentiated when cobalt is included, we would like to present the results obtained in our laboratories confirming this earlier work.

LITERATURE CITED

Pratt and Dufrenoy[4] were the first to notice that trace amounts of cobalt salts increased the *in vitro* antibacterial activity of penicillin against both gram-positive and gram-negative organisms. They reported[5,9] that cobalt enhanced the activity of penicillin from two to ten times against *Micrococcus pyogenes* var. *aureus* (Staphylococcus), *Escherichia coli*, *Bacillus subtilis*, *Proteus vulgaris*, and *Eberthella typhosa*. Pital *et al.*[10,11] reported comparable *in vitro* results when cobalt was combined with penicillin against *Salmonella pullorum*. Kammerer and Eberle[12] found cobalt potentiated penicillin fourfold against *Micrococcus pyogenes* var. *aureus* and streptomycin and sulfisoxazole twofold against *Escherichia coli* and *Micrococcus pyogenes* var. *aureus*, respectively, using a standard tube dilution method. They noticed no benefit when cobalt was combined with chloramphenicol (chloromycetin®) or oxytetracycline (terramycin®), and actual interference with the activity of chlortetracycline (aureomycin®) and protocid® (a combination of sulfanilamide, sulfadiazine, and sulfaethylthiadiazole). The data of Erszsebet and Tivadar[13] demonstrated that cobalt reduced the amount of streptomycin required to inhibit visible growth from two to eight times against seven strains of coliform organisms in a tube dilution method. The work of Carboncini[14] indicated a two- to fourfold enhancement by combining penicillin and cobalt *in vitro* against *Micrococcus pyogenes* var. *aureus* and *Escherichia coli*.

Many of these same workers have reported similar *in vivo* activity when using a cobalt-penicillin combination. Strait *et al.*[5] reported 2,000 units of penicillin with cobalt to be at least equally effective as 4,000 units of penicillin alone in mice inoculated with *Eberthella typhosa*. Carboncini[14] repeated this work with comparable results. Pital and co-workers[10,11] found that cobalt added to the drinking water materially increased the value of penicillin in treating pullorum infected chicks; 2,290 units of penicillin plus cobalt was more than twice as effective as 5,500 units of penicillin alone. Carboncini[14] and Liguori[16] found it difficult to estimate the comparative benefit of penicillin-cobalt therapy for infections in man, but many cases recovered which had been previously resistant to penicillin.

METHODS

Single Antibiotics and Cobalt.—A simple screening technique was adopted. To determine which antibiotics would be augmented by the addition of cobalt, 18 cc. of standard nutrient agar was placed in sterile Petri dishes and allowed to solidify, then 3 cc. of aqueous $CoCl_2 \cdot 6H_2O$ 1:5,000 was placed on the agar for surface penetration for forty-five minutes and was then decanted. A group of control agar plates was treated with 3 cc. of sterile distilled water using a similar technique. The plates were then streaked with cotton swabs with a 24-hour tryptose phosphate broth culture of *Micrococcus pyogenes* var. *aureus* strain H-263-2 isolated from a clinical case of mastitis. One Difco antibiotic disk was placed in each quadrant, the plates were inverted and incubated for eighteen hours at 37 C. The diameters of the zone of inhibition of the cobalt-treated plates were compared with those in the control plates.

Antibiotic Combination and Cobalt.—One loopful of a 24-hour tryptose phosphate broth culture of the strain being tested was transferred to a second tube of tryptose phosphate broth. One loop from this 10 cc. diluted broth culture was in turn transferred to a third tube of tryptose phosphate broth; 0.5 cc. of the final culture was combined with 0.5 cc. of tryptose phosphate broth contain-

From the Fort Dodge Laboratories, Inc., Fort Dodge, Iowa.

TABLE 1—The Effect of Cobalt in Vitro on the Antibiotic Activity Against Micrococcus Pyogenes var. Aureus

	Penicillin (units)			Streptomycin (μg.)			Bacitracin (units)			Polymyxin (units)			Chlortetracycline (μg.)			Oxytetracycline (μg.)			Chloramphenicol (μg.)		
	.5	1	10	1	10	100	2	10	20	50	100	300	10	30	60	10	30	60	10	30	60
No cobalt added	*20.8	22.5	33.5	.....	12.5	18.2	9.2	12.3	14.0	.....	.....	9.0	17.0	17.5	19.0	12	16	17.0	13.5	**	22.0
(3 cc. distilled H_2O)																					
Cobalt added	22.7	24.3	35.8	.....	12.8	19.3	11.0	13.6	16.3	.....	.....	9.0	17.0	17.5	19.0	12	16	17.0	13.5	**	22.0
(3 cc. $CoCl_2 \cdot 6H_2O$)																					

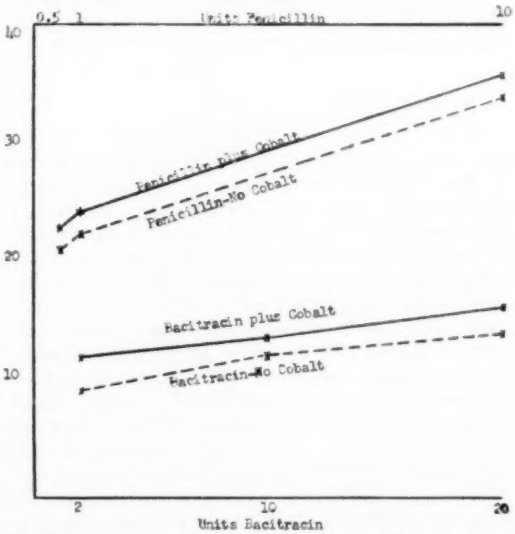
* Represents the diameter of zones of inhibition in millimeters; the average of three plate determinations. ** Not determined.

Diameters of zones of inhibition corresponding to different concentrations of Difco penicillin and bacitracin disks. The standard nutrient agar plates were seeded with a 24-hour culture of *Micrococcus pyogenes* var. *aureus*, H-263-2, after a 45-minute exposure of $CoCl_2$ (1:5,000) or distilled water.

ing: (A) graduated amounts of penicillin and streptomycin or (B) graduated amounts of penicillin and streptomycin plus 2.5 mg. per 100 cc. each of sulfisoxazole and sulfathiazole or (C) graduated amounts of penicillin, streptomycin, 2.5 mg. per 100 cc. each of sulfisoxazole and sulfathiazole and 0.333 μg. of $CoSO_4 \cdot 7H_2O$. Positive results were considered to be those in which no visible turbidity developed in eighteen hours when incubated at 37 C.

RESULTS

Single Antibiotics and Cobalt.—Cobalt was found to stimulate the inhibitory activity of penicillin, streptomycin, and bacitracin but had no action on chloramphenicol, chlortetracycline, and oxytetracycline. The results are presented in table 1 using *Micrococcus pyogenes* var. *aureus* H-263-2 as the test organism. The calculated degree of enhancement with penicillin and bacitracin is shown in chart 1. Since three antibiotic levels were used, only the percentage of potentiation of the lower two concentrations can be estimated. The cobalt-enhancing factor when penicillin was used was 2.4 for both the 0.5-unit and the 1.0-unit disks. That is, each unit of penicillin with cobalt was equivalent to 2.4 units of penicillin alone. Since streptomycin had activity against *Micrococcus pyog-*



Diameters of zones of inhibition corresponding to different concentrations of Difco penicillin and bacitracin disks. The standard nutrient agar plates were seeded with a 24-hour culture of *Micrococcus pyogenes* var. *aureus*, H-263-2, after a 45-minute exposure of $CoCl_2$ (1:5,000) or distilled water.

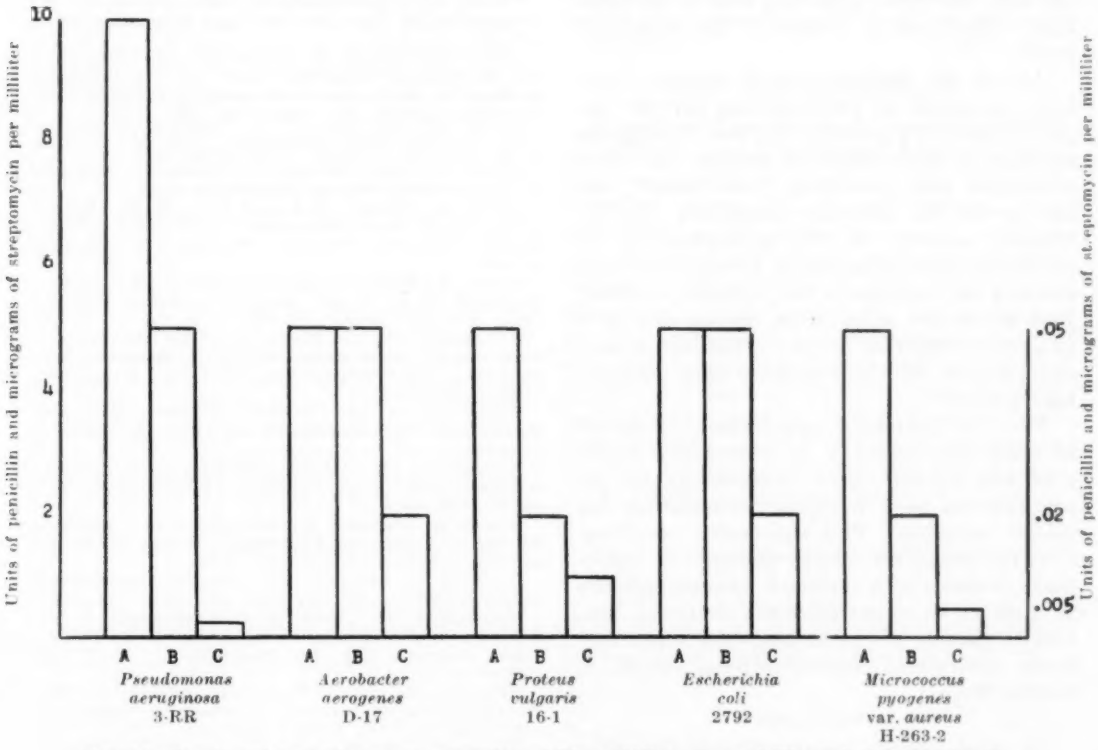
Chart 1—The effect of cobalt with penicillin and bacitracin.

enes var. *aureus* only at the two higher concentrations (10 and 100 μg.) it was not included in table 1. The enhancing factor with bacitracin was 3.3 for 2 units and 1.8 for 10 units. Similar work resulted in potentiation of penicillin against *Micrococcus pyogenes* var. *aureus* FDA 209, and *Salmonella choleraesuis*. The action of streptomycin likewise was benefited by cobalt against *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, and *Salmonella choleraesuis*.

Antibiotic Combination and Cobalt.—Cobalt increased the antibiotic action of the combination of penicillin, streptomycin, sulfisoxazole, and sulfathiazole. Chart 2 shows the comparative minimum inhibitory levels of the three combinations used. The right ordinate of chart 2 is for the *Micrococcus pyogenes* var. *aureus* only, since this organism was more susceptible to the antibiotic combination than the other four species. Penicillin and streptomycin alone (A) become approximately twice as effective when the sulfonamides were added against *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Micrococcus pyogenes* var. *aureus* (B). On the other hand, sulfisoxazole and sulfathiazole did not increase the effectiveness of penicillin-streptomycin against *Aerobacter aerogenes* or *Escherichia coli*. When 0.333 μg. of cobalt (C) was added to the same combination of penicillin, streptomycin, sulfisoxazole, and sulfathiazole, the bacteriostatic effectiveness was from at least two to ten times greater against all five strains tested.

DISCUSSION

At the present time, one can only theorize concerning the probable mode of action of cobalt. The *in vitro* results in our laboratory as well as those published by other workers indicate cobalt used alone in low concentrations is not bacteriostatic. Some workers [5,14] originally advanced the theory that cobalt stimulated bacterial multiplication and since penicillin is more



Concentration in units or micrograms preventing visible turbidity. A—penicillin and streptomycin; B—penicillin, streptomycin plus sulfathiazole and sulfisoxazole, 2.5 mg. per 100 cc. of each; C—same as B but with 0.333 μg. of cobalt sulfate per milliliter of each.

Chart 2—Improved bacterial inhibition produced when cobalt was added to a combination of antibiotics and sulfonamides.

effective against dividing bacteria, its bactericidal action would be enhanced in the presence of cobalt.

More recently Pital *et al.*[11] reported cobalt *in vitro* inhibits the normal respiration of bacteria. Other workers[17,20] have stated cobalt is capable of combining with the sulfhydryl groups which are active in oxygen transfer. Therefore, cobalt would interfere with normal respiration and utilization of the essential amino acids by the organism. For example, cobalt probably combines with glutathione, a sulfhydryl containing amino acid which must be obtained by the bacterial cell from its environment. This amino acid is structurally similar to penicillin and, when cobalt combines with it, the penicillin may be selectively utilized by the cell and thus could become more effective at a lower concentration.

Cobalt is also reported to block nucleic acid metabolism in at least one species of bacteria.[17] Since penicillin and streptomycin also interfere with this step in metabolism, cobalt could augment the action of both.

All of the forementioned theories have been proposed as explanations for the improved *in vitro* results. Liguori[16] suggests another possible mode of action. He demonstrated that penicillin plus cobalt, unlike penicillin, actually augments the defensive activity of the granulocytes by elevating their phagocytic index as well as causing an increase in the number. Cobalt, just as do the antibiotics, apparently acts in several different ways so that one or any combination of these actions may actually take place.

The first practical application of cobalt in antibiotic therapy in veterinary medicine has already been realized by its incorporation in a mastitis formulation for udder infusion. The successful combination of penicillin-cobalt therapy in infectious diseases of a series of human patients as well as in experimentally induced bacterial disease of mice and chickens suggests additional investigational work is warranted.

CONCLUSION

In vitro results presented indicate that cobalt enhances the antibacterial effectiveness of penicillin, streptomycin, and bacitracin against *Micrococcus pyogenes* var. *aureus*. There was no benefit noted when cobalt was combined with oxytetracycline, chlortetracycline, chloramphenicol, or polymyxin. In a tube dilution method, cobalt reduced the inhibiting level of a combination of penicillin, streptomycin, sulfisoxazole, and sulfathiazole from two- to tenfold against *Micrococcus pyogenes* var. *aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Aerobacter aerogenes*, all organisms isolated from clinical mastitis.

References

[1] Bosworth, T. J.: Control of Livestock Diseases in Britain: Some Reflections on the Present Situation. Proc. Roy. Soc. Med., *47*, (1954): 23-26.

[2] Edds, G. T., Roepke, M. H., and Bieter, R .N.: Effect of Certain Drugs Alone and In Combination on Micrococci Isolated from the Bovine Udder. Am. J. Vet. Res., *14*, (1953): 344-361.

[3] Hancock, B. B., Edds, G. T., and Bromelkamp, E. T.: Control and Treatment of Mastitis. Iowa Vet., *24*, (1953): 20-26.

[4] Pratt, R., and Dufrenoy, J.: Cytochemical Mechanisms of Penicillin Action. IV. Comparative Response of Gram-Positive and Gram-Negative Bacteria to Penicillin. J. Bact., *54*, (1947): 719-730.

[5] Strait, L. A., Dufrenoy, J., Pratt, R., and Lamb, V.: Enhancement of Penicillin Effectiveness by Traces of Cobalt. J. Am. Pharm. A., Sci. ed., *37*, (1948): 133-135.

[6] Pratt, R., and Dufrenoy, J.: Recent Research on Penicillin Action. Texas Rep. Biol. and Med., *7*, (1949): 180-219.

[7] Pratt, R., Dufrenoy, J., and Strait, L. A.: Cytochemical Mechanism of Penicillin Action. VI. The Influence of Cobalt on the Optimal Bacteriostatic Concentration of Penicillin. J. Bact., *55*, (1948): 727-738.

[8] Pratt, R., and Dufrenoy, J.: Antibiotics. 1st ed. J. B. Lippincott Company, Philadelphia, 1949.

[9] Pratt, R., and Dufrenoy, J.: Antibiotics. 2nd ed. J. B. Lippincott Company, Philadelphia, 1953.

[10] Pital, A., Stafseth, H. J., and Lucas, E. H.: Observations on the Cobalt Enhancement of Penicillin Activity Against *Salmonella Pullorum*. Science, *117*, (1953): 459-460.

[11] Pital, A., Stafseth, J. J., and Lucas, E. H.: An Experimental Study on the Control of Pullorum Disease. Poult. Sci., *33*, (1954): 134-139.

[12] Kammerer, H., and Eberle, A.: Uber Die Verstarkung Mancher Antibiotischer Substanzen, Besonders Des Penicillins, Durch Kobalt. Klin. Wchnschr., *45* and *46*, (1952): 1083-1086.

[13] Erszsebet, O., and Tivadar, C.: Streptomycin es Nehezezfemek Synergismusa, Orvosi hetil., *93*, (1952): 1251-1252.

[14] Carboncini, G.: Controlle Dell'Efficacia Terapeutica Dell'Associazone Cobalte-Penicillina. Minerva med., *42*, (1951): 396-398.

[15] Pratt, R., Dufrenoy, J., and Strait, L. A.: The Enhancement of Penicillin Effectiveness *in Vivo* by Traces of Cobalt. J. Bact., *55*, (1948): 75-77.

[16] Liguori, A.: Ricerche-Sperimentale Sull'Associazione Cobalto-Penicillina. Minerva med., *43*, (1952): 149-152.

[17] Abelson, P. H., Aldons, E.: Ion Antagonisms: Interference of Normal Magnesium Metabolism by Nickel, Cobalt, Cadmium, Zinc, and Manganese. J. Bact., *60*, (1950): 401-413.

[18] Calvin, I. M., Bailes, R. R., and Wilmarth, W. K.: The Oxygen Carrying Synthetic Chelate Compounds. J. Am. Chem. Soc., *68*, (1946): 2254-2256.

[19] Schmidt, J. R., and Rasmussen, A. F., Jr.: Inhibitory Effect of Cobaltous Ions on the Multiplication of Influenza Virus. Proc. Soc. Exptl. Biol. and Med., *81*, (1952): 244-246.

[20] Schade, A. L.: Cobalt and Bacterial Growth, With Special Reference to *Proteus Vulgaris*. J. Bact., *58*, (1949): 811-822.

The Effects of a Chlorophyll Derivative when Included in a Ration Fed Rats
II. Reproduction, Blood, and Tissue Studies

ELWOOD F. REBER, Ph.D., and D. A. WILLIGAN, D.V.M.

Urbana, Illinois

Chlorophyll fractions deserve investigation because they are of economic importance to the practicing veterinarian, the manufacturer of proprietary drugs and dog foods, as well as to the consumer. The effect of sodium potassium copper chlorophyllin, when included in a ration and fed to rats continuously during the growth and reproduction period, is reported here.

Literature Cited

Reports of Serling,[1] Westcott,[2] and Beggs[3] indicate that chlorophyll fractions administered orally aided in eliminating most objectionable body and mouth odors. These workers also considered chlorophyll fractions as nontoxic and as having no effect on normal digestive activities when fed for short periods of time. Worden and Waterhouse[4] found that 33 mg. of water-soluble chlorophyll given daily was effective in controlling halitosis in dogs, but 33 mg. given four or six times daily had a negligible effect on female sex odor and did not influence mating. Burkhardt and Phillips[5] reported a dose of 100 mg. of a chlorophyll fraction given to female dogs morning and night was sufficient to eliminate the attraction for male dogs.

Methods

The rats used in this study were the Sprague-Dawley strain. Six female rats, about 28 days old, were allotted to each treatment. The initial average weight of the rats in each treatment was 43 Gm. The control group was fed the basal ration whose composition is shown in table 1. The second group received the basal ration in which Na K Cu chlorophyllin* (potassium disodium copper salt having the formula $C_{34}H_{31}O_6N_4Cu\,K\,Na_2$) was included at the expense of sucrose, at a level equivalent to 1 per cent of the pure compound. The Na K Cu chlorophyllin had a potency of 75 per cent.

The rats were individually caged and housed in a room maintained at 25.5 ± 1 C. They were weighed at weekly intervals and, while fed *ad libitum*, food consumption records were maintained for the initial ten-week growth period. Mating of both groups of females was started at the end of the eleventh experimental week (about 15 weeks of age). One male was caged with each female for one week with rotation of males for three consecutive matings of one week each. The females were then separated from the males.

Blood samples were taken by snipping the end of the tail of the parent-generation rats. The blood was used for the determination of hemoglobin by the acid hematin method,[6] total white blood cell count, and the differential count of Schilling.[7] Suitable smears were prepared and stained with Wright's stain. In the study, a total of 200 cells was the basis for the differential count of Schilling.

Results and Discussion

Growth.—The effect of the chlorophyll derivative upon growth and food efficiency has been briefly presented by the authors.[8] Detailed information on growth and food efficiency for the parent generation and the first litters of the parent generation is presented in tables 2 and 3. Under the conditions of this experiment, Na K Cu chlorophyllin included in the ration was neither stimulatory nor inhibitory with respect to growth and feed efficiency during the first ten weeks of growth. After being fed the experimental rations for nineteen weeks, the body weight of the control females averaged 217 Gm. and the group fed the Na

TABLE 1—Composition of the Basal Ration

Ingredients	Per cent
Crude casein	25.0
Wheat germ oil (2 I.U. vit. E/Gm.)	3.0
Cod liver oil (1,000 U.S.P. A, 100 U.S.P. D/Gm.)	2.0
Salt (U.S.P. XIV)	4.0
Vitamin mix*	1.2
Lard	5.0
Sucrose	59.8

* Vitamin mix supplied per kilogram of ration: thiamine, 8 mg.; riboflavin, 8 mg.; pyridoxine, 5 mg.; inositol, 1 Gm.; para-aminobenzoic acid, 0.3 Gm.; biotin, 250 μg.; folic acid, 500 μg.; calcium pantothenate, 100 mg.; choline, 3 Gm.; nicotinic acid, 8 mg.

From the College of Veterinary Medicine and the Agricultural Experiment Station, University of Illinois, Urbana.

The authors express their appreciation to Dr. H. H. Draper, Merck and Company, Rahway, N. J., for supplying B vitamins; to Dr. C. C. Morrill and Miss Marian Norwich, Department of Veterinary Pathology and Hygiene, University of Illinois, Urbana, for their aid and advice; and to Dr. H. W. Norton, University of Illinois, Urbana, for the statistical analyses.

* Purchased from Archer-Daniels-Midland Co., Chlorophyll Division, R. D. 1, Bethlehem, Pa., and Neodesha, Kan.

K Cu chlorophyllin in the ration averaged 228 Gm. The growth curves for the two groups were approximately the same.

Reproduction.—All the females in both groups conceived during the first three-week mating period. Table 3 presents the data regarding the first litters of the parent generation. The inclusion of Na K Cu chlorophyllin in the ration apparently did not influence the number of young conceived and born. One female in this group died soon after successfully whelping 11 live rats. The death of the female necessitated the destruction of the 11 young which in turn decreased the number of possible young weaned as shown in table 3. The young rats of three litters all died within the first five days following whelping. Therefore, of the six litters born to the Na K Cu chlorophyllin group only two survived to weaning age (21 days). This low survival rate, together with the average weaning weight of 30 Gm. for the weanlings compared to 40 Gm. weaning weight for the controls, suggests Na K Cu chlorophyllin in some way interfered with normal livability of the young rats and/or the normal lactation ability of the females. Following weaning, the young rats were fed the same ration as their parents.

Under these conditions, the Na K Cu chlorophyllin first-generation group had an average weight at 28 days of age of 48 Gm., a gain of 18 Gm. in seven days as compared to 53 Gm., a gain of 13 Gm., for the control group, thus indicating the factor responsible for the poor growth which occurred during the lactation period apparently was not present or did not affect growth during the seven-day postweaning period. However, during this period 4 of the weanlings from the Na K Cu chlorophyllin group did show weakness and poor coordination of the hind legs so that they dragged their rear legs as they moved about the cage. These symptoms occurred only during the first two to three days following weaning.

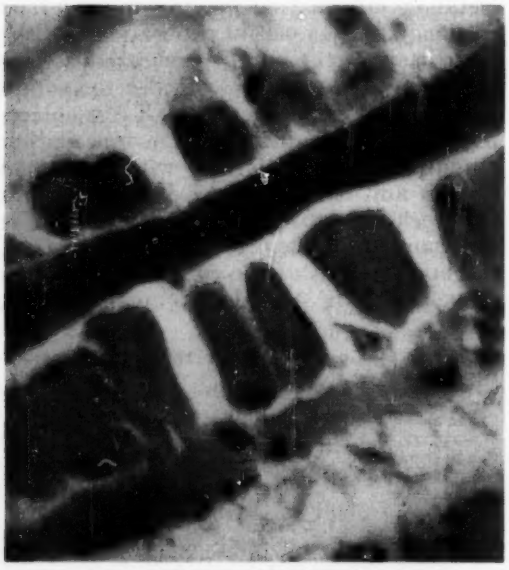


Fig. 1—Row of dark-staining nuclei within substance of muscle fiber. Van Gieson's stain. x 430.

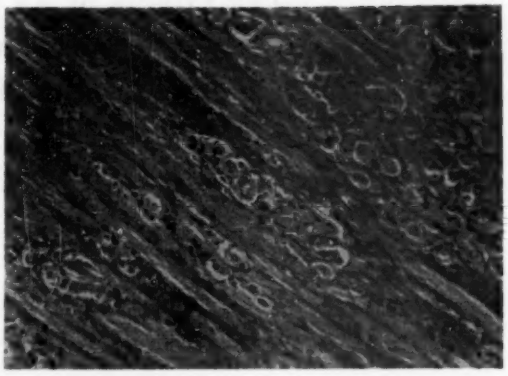


Fig. 2—Relatively normal muscle fibers surrounded by areas of active phagocytosis of previously degenerated cells. Van Gieson's stain. x 80.

With respect to the early loss of young by the group of females fed the Na K Cu chlorophyllin, it is interesting to note the work of Cunningham. Cunningham [9] reported normal breeding and whelping by those females fed copper acetate, but no litters were produced by those females receiving verdigis (basic copper acetate) when added to the ration to supply 7.5 mg. of copper per day per rat. These compounds were not toxic to the adult rat.

If the Na K Cu chlorophyllin is assumed to be the potassium disodium copper salt having the formula $C_{34}H_{31}O_6N_4Cu\,K\,Na_2$, it would have the molecular weight of 740. The inclusion of this compound at a level of 1 per cent in the diet would supply a level of 0.87 mg. of copper per gram of food. The rats ate an average of 9.4 Gm. of diet per day during the first ten weeks.

TABLE 2—Average Weight Gain and Feed Efficiency of the Parent-Generation Rats

	Control			Na K Cu chlorophyllin		
	Ave.		Range	Ave.		Range
Ten-week weight gain (Gm.)	188	(6)*	166 to 211	192	(6)	178 to 211
Food consumed/weight gain (10 weeks)	3.50	(6)	3.33 to 3.72	3.44	(6)	3.19 to 3.67
Nineteen-week weight gain (Gm.)	217	(6)	207 to 233	228	(5)	202 to 255

* Represents number of animals in the group.

This would have been an average intake of 8.1 mg. of copper per rat per day.

Hematology.—The results of the white blood cell counts are shown in table 4. The total leukocyte values and the differential counts of Schilling were within the range of "standard values" as described by Albritton [10] for both the control animals and those fed chlorophyllin. However, the rats fed the ration containing Na K Cu chlorophyllin had an average neutrophil segmented cell count significantly greater (at the 1 % level) than the respective count of the control group. The increased number of neutrophil cells were responsible for a higher total leukocyte count found in the rats fed chlorophyllin than in the control group.

The average hemoglobin level for both groups was the same at 14 Gm. per 100 cc. of blood. Polychromasia and normoblasts were observed in the blood smears of all rats in the control group. These cells were observed in only 1 of 5 of the animals fed the ration containing Na K Cu chlorophyllin. Cunningham [9] has reported a possible effect of copper to be an increase in "organic iron" achieved by the preliminary formation of a copper porphyrin and subsequent replacement of the copper by iron. Smith [11] has expressed the view, based on limited experimental evidence, that Na K Cu chlorophyllin might have a stimulating

TABLE 3—Data Concerning First Litters of Parent-Generation Rats

	Control	Na K Cu chlorophyllin
No. young born	59	54
No. young weaned	49	17
Ave. weight of weanlings (Gm.)	40	30
Ave. 28-day weight (Gm.)	53	48
Ave. 7-day gain in weight (Gm.)	13	18

effect upon hematopoiesis as well as a stimulating effect upon the cells of the reticuloendothelial system. If a copper porphyrin is an intermediate in hemoglobin formation, as Cunningham suggested, it is possible that the Na K Cu chlorophyllin stimulates the rate or alters the pathway of hemoglobin and/or erythrocyte formation and thus accounts for the absence of polychromasia and normoblasts.

Morbid Anatomy.—Six weanling rats fed the basal ration and 9 fed the ration containing the Na K Cu chlorophyllin were destroyed at 28 days of age. The hair of all the rats fed the ration containing Na K Cu chlorophyllin was rough and pale green in color. The skeletal musculature of the whole body possessed a greenish hue thus indicating a general systemic distribution

TABLE 4—Total and Differential White Blood Cell Counts of Parent-Generation Rats

Elements	Control		Na K Cu Chlorophyllin	
	Absolute	%	Absolute	%
Basophils	25	0.2	29	0.2
Eosinophils	62	0.5	231	1.6
Neutrophil band cells	86	0.7	29	0.2
Neutrophil segmented cells	1,917*	15.5	4,104*	28.4
Lymphocytes	9,744	78.8	9,595	66.4
Monocytes	532	4.3	462	3.2
Total	12,366	100	14,450	100

* Significantly different at the 1 per cent level.

of the chlorophyll derivative. Antopol and Glaubach [12] reported the muscles of mice were distinctly green six months after injection of 5 to 25 mg. of Na K Cu chlorophyllin. The marked pallor of several hindleg, foreleg, sublumbar, and/or masseter muscles of the weanling rats was of interest. Not all of the rats consistently showed degeneration of all muscles mentioned. There was no obvious pattern or order of degeneration. Of interest also was the intense green color of the esophageal portion of the stomach in all test animals. The discoloration was very noticeable even through the serosa. The livers were found to be consistently paler in color but normal in size, shape, and consistency. The average liver weight, calculated as percentage of body weight, was 6.9 for the group fed the Na K Cu chlorophyllin ra-

tion and 6.1 for the group fed the basal ration.

Histopathological examination of the degenerated skeletal muscles taken from 28-day-old first-generation rats revealed changes consisting of swelling of the muscle fibers with subsequent hyalinization. Occasionally, a muscle fiber without any discernible swelling appeared to contain a row of dark-staining nuclei arranged within the substance of the fiber and parallel to its long axis. No mitoses were apparent (fig. 1). Forbus [13] considers the row of dividing nuclei as the first evidence of degeneration in a field of otherwise normal muscle. Areas of marked degeneration and hyalinization showed evidence of very active phagocytosis of remnants of the necrotic muscle by multinucleated cells. Leukocytes were conspicuously absent. In some of the affected areas, relatively normal fibers (fig. 2) were observed surrounded by areas of active phagocytosis of previously degenerated cells. The picture is one of apparent selective degeneration of certain muscle fibers. Tissue sections stained by Van Gieson's method [14] indicated no evidence of fibrosis in and around the degenerated foci of muscle.

SUMMARY

The effects of including Na K Cu chlorophyllin at a level equivalent to 1 per cent of a 100 per cent potency in a ration fed to rats has been explored. With 6 females per treatment it was found, under the conditions of this expermient, that Na K Cu chlorophyllin did not influence growth or affect the rate of conception or the number of young born. The Na K Cu chlorophyllin adversely affected the survival of the first-generation rats during the first five days following birth and adversely affected lactation as measured in terms of the weight of the young rats at weaning age. The inclusion of Na K Cu chlorophyllin in the ration of the parent generation caused a significant increase in neutrophils. Hemoglobin values were 14 Gm. per 100 cc. of blood for each group, and the inclusion of Na K Cu chlorophyllin decreased the incidence of polychromasia and normoblasts. First-generation weanling rats continued on Na K Cu chlorophyllin ration showed some locomotory difficulties, although their growth rate appeared to be satisfactory up to 28 days of age. Postmortem examination of these rats indicated a general distribution of the chlorophyll derivative throughout the skeletal muscles. Histological examination revealed an apparent selective degeneration and phagocytosis of affected muscle fibers.

References

[1] Serling, M. E.: Control of Body and Breath Odors with Chlorophyll Fractions. Vet. Med., *45*, (1950): 291-292.

[2] Westcott, H. F.: Oral Chlorophyll Fractions for Body and Breath Deodorization. New York State J. of Med., *50*, (1950): 698-700.

[3] Beggs, W. E.: The Effectiveness of Some Chlorophyll Derivatives in the Control of Canine Mouth Odors. J. Small Anim. Med., *1*, (1952): 215-219.

[4] Worden, A. N., and Waterhouse, C. E.: A Note on the Oral Administration of Chlorophyll to the Dog. Vet. Rec., *64*, (1952): 573-574.

[5] Burkhardt, B. S., and Phillips, L. R.: Control of Canine Estrual Odor with Chlorophyll. J. Small Anim. Med., *1*, (1952): 47-49.

[6] Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry. The Blakiston Co., Philadelphia. 1949.

[7] Hepler, O. E.: Manual of Clinical Laboratory Methods. Charles C. Thomas, Springfield, Illinois. 1951.

[8] Reber, E. F., and Willigan, D. A.: The Effects of Sodium Potassium Copper Chlorophyllin when Included in the Ration of the Rat. I. Growth (abstr.). Fed. Proc., *13*, (1954): 279.

[9] Cunningham, I. J.: Some Biochemical and Physiological Aspects of Copper in Animal Nutrition. Biochem. J., *25*, (1931): 1267-1294.

[10] Albritton, E. C.: Standard Values in Blood. W. B. Saunders Co., Philadelphia. 1952.

[11] Smith, L. W.: Chlorophyll: An Experimental Study of its Water-Soluble Derivatives. I. Remarks Upon History, Chemistry, Toxicity, and Antibacterial Properties of Water-Soluble Chlorophyll Derivatives as Therapeutic Agents. Am. J. M. Sci., *207*, (1944): 647.

[12] Antopol, W., and Glaubach, S.: Pathology of Chlorophyll Administration in Mice (abstr.). Fed. Proc., *13*, (1954): 421.

[13] Forbus, Wiley D.: Pathologic Changes in Voluntary Muscle. I. Degeneration and Regeneration of the Rectus Abdominis in Pneumonia. Arch. Path. and Lab. Med., *2*, (1926): 318-339.

[14] Cowdry, E. V.: Laboratory Technique in Biology and Medicine. The Williams and Wilkins Company, Baltimore. 1952.

Response of the Cave Bat (*Myotus Lucifugus*) to the "B" Virus

REGINALD L. REAGAN, Major (Ret.), U.S.A.; EDWARD C. DELAHA, B.S.; A. L. BRUECKNER, V.M.D.

College Park, Maryland

THE "B" virus was isolated from the spleen and central nervous system of patient "B" and maintained since the original isolation in 1932 by serial passages in rabbits.[2] The virus is extremely pathogenic for rabbits by most routes of inoculation. Following intracutaneous injection, a hemorrhagic necrotic area develops around the site of inoculation. Sabin and Wright [3] found that one to two days before the onset of paralysis, most rabbits exhibit a tendency to bite the local lesions.

This experiment was conducted to determine if the cave bat is susceptible to the virus, as the bat was found to be susceptible to rabies in a previous experiment by Reagan *et al.*,[1] and to determine if the cave bat would harbor the "B" virus.

MATERIALS AND METHODS

The "B" virus (strain 1) used for this study was furnished by the American Type Culture Collection of Washington, D. C. Before receipt at this laboratory the virus had been passed 67 times in rabbits. Lyophilized rabbit spinal cords of the sixty-seventh passage were received at this laboratory and were diluted to a 5 per cent suspension with physiological saline.

One 3-lb. albino rabbit was inoculated intradermally with 0.1 ml. of the 5 per cent suspension. The rabbit showed paralysis and nervous symptoms seven days postinoculation. This confirmed the original virus, obtained from the lyophilized material, to be viable. The balance of the viable suspension was used to initiate the following experiment in the cave bats.

Forty-four healthy cave bats (young, but age unknown) were divided into 11 groups of 4 each. The bats of each group (except a control group) were administered the virus by one of the following routes: intracerebral, intraperitoneal, intradermal, intracardial, intramuscular, intralingual, intraoral, intrarectal, intraocular, and subcutaneous. In all routes of exposure, the inoculum per bat consisted of 0.03 ml. of the 5 per cent suspension except the rectal route which consisted of 0.1 ml. of inoculum per bat. The bats were housed in mouse jars covered with a wire lid (4 bats to each jar). Each jar was supplied daily with 1 tablespoonful of a mixture containing cottage cheese, bananas, meal worms, and vitamins and a small jar of water. Two paper towels also were placed in each jar for the bats to use for clinging.

RESULTS AND DISCUSSION

Table 1 shows the response of bats infected with "B" virus by different routes of exposure. After fourteen days of exposure, only bats from the intracerebral group showed signs of nervous system involvement. Bats from each exposed group were destroyed and their brains aseptically removed and pooled. The pools were ground separately with physiological saline to a 20 per cent suspension and 1 rabbit was injected intracerebrally with each pool. The rabbit injected with the suspension from the intracerebral group showed nervous symptoms within seven days. No signs of nervous system involvement were manifested by the rabbits injected with the suspensions from the other groups.

Bat brain suspension from the intracerebral group was injected into a rabbit which had been immunized against "B" virus and into a normal rabbit. The latter showed symptoms of nervous system involvement, while the immunized rabbit showed no ill effect. This confirmed the virus of the intracerebral suspension to be "B" virus.

TABLE 1—Response of Cave Bats to the "B" Virus

Route of inoculation	No. of bats exposed	No. showing symptoms	Minimum-maximum incubation period* (days)
Intracerebral	4	4	10
Intraperitoneal	4	0	
Intradermal	4	0	
Intracardial	4	0	
Intramuscular	4	0	
Intralingual	4	0	
Intraoral	4	0	
Intrarectal	4	0	
Intraocular	4	0	
Subcutaneous	4	0	
Normal controls	4	0	

*Incubation period—time between exposure to virus and onset of symptoms.

From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park; professor of virus diseases (Reagan), virologist (Delaha), professor and director (Brueckner).

The authors acknowledge the technical assistance of Miss Carolyn J. Biehn in this experiment.

SUMMARY

This experiment showed the cave bat *(Myotus lucifugus)* to be susceptible to the "B" virus by intracerebral inoculation. Cave bats exposed to the "B" virus by intraperitoneal, intradermal, intracardial, intramuscular, intralingual, intraoral, intrarectal, intraocular, and subcutaneous routes of inoculation evidenced no symptoms of central nervous system involvement. Brain suspensions of all of the bats except those intracerebrally exposed, when injected intradermally into rabbits, failed to produce symptoms of central nervous system involvement.

References

[1] Reagan, R. L., Delaha, E. C., and Brueckner, A. L.: Response of the Cave Bat to Several Strains of Rabies Virus. To be published, 1954.

[2] Rivers, T. M.: Viral and Rickettsial Infections of Man. J. B. Lippincott Co., Philadelphia (1949): 198.

[3] Sabin, A. B., and Wright, A. M.: Acute Ascending Myelitis Following a Monkey Bite, With the Isolation of a Virus Capable of Reproducing the Disease. J. Exptl. Med., *59*, (1934): 115.

COPYRIGHT 1954 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

Printed in the U.S.A